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TECHNIQUE OF ORGANIC CHEMISTRY

ARNOLD WEISSBERGER, *Editor*

Volume X

FUNDAMENTALS OF CHROMATOGRAPHY

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Volume I:	Physical Methods of Organic Chemistry *Second Edition* Parts I to III
Volume II:	Catalytic, Photochemical, and Electrolytic Reactions *Second Edition*
Volume III:	*Second Edition* Part I. Separation and Purification Part II. Laboratory Engineering
Volume IV:	Distillation
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Volume VIII:	Investigation of Rates and Mechanisms of Reactions
Volume IX:	Chemical Applications of Spectroscopy
Volume X:	Fundamentals of Chromatography

TECHNIQUE OF ORGANIC CHEMISTRY
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FUNDAMENTALS OF CHROMATOGRAPHY

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INTRODUCTION

Organic chemistry, from its very beginning, has used specific tools and techniques for the synthesis, isolation, and purification of compounds, and physical methods for the determination of their properties. Much of the success of the organic chemist depends upon a wise selection and a skillful application of these methods, tools, and techniques, which, with the progress of the science, have become numerous and often intricate.

The present series is devoted to a comprehensive presentation of the techniques which are used in the organic laboratory and which are available for the investigation of organic compounds. The authors give the theoretical background for an understanding of the various methods and operations and describe the techniques and tools, their modifications, their merits and limitations, and their handling. It is hoped that the series will contribute to a better understanding and a more rational and effective application of the respective techniques.

The field is broad and some of it is difficult to survey. Authors and editor hope that the volumes will be found useful and that many of the readers will let them have the benefit of their criticism and of suggestions for improvements.

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A. W.

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Volume X. **Fundamentals of Chromatography.** H. G. Cassidy

FUNDAMENTALS OF CHROMATOGRAPHY

EDITOR'S PREFACE

Volume V of this series, *Adsorption and Chromatography*, by Professor H. G. Cassidy, was planned to be a general treatise on adsorption, of which chromatography is a part; about one-third of the volume deals especially with chromatography. In recent years great progress has been made in chromatography so that the earlier discussion has become inadequate, but relatively less of a change has taken place in other areas of adsorption.

Rather than reprinting the original discussion of these areas of adsorption together with a new treatment of chromatography, the publishers are keeping the original Volume V available for readers interested in the topics of Chapters I to V (Definitions, Molecular Aspects of Adsorption; Measurement of Adsorption, Treatment of Data; Relations between Relative Adsorbability and Properties of Phases; Relations between Relative Extent of Adsorption and Properties of Adsorptive; and Graded Eluents and Adsorbents), while chromatography is discussed from new points of view in the present volume as stated in the author's preface.

A.W.

FUNDAMENTALS OF CHROMATOGRAPHY

AUTHOR'S PREFACE

The literature of chromatography has now reached vast proportion. In 1954 Dr. I. M. Hais and Dr. K. Macek, in their treatise *Papírová Chromatografie* listed 3795 references, and by now probably several thousand more papers have appeared. Papers on adsorption chromatography must be fully as numerous, and publications in the field of gas chromatography are multiplying at a rapid rate. The situation in a related area of importance to chromatography is indicated by the two volumes of Dr. V. R. Deitz's *Bibliography of Solid Adsorbents* (1900–1942; 1943–1953) in which 19,765 papers are abstracted, and three more years of active publication have since passed.

Faced with such a stupendous literature, I concluded, with the Editor's approval, that in this book I should approach chromatography at the level of principle, and not attempt to write a detailed review or compendium of methods. In my experience people who seek aid in solving chromatographic problems are generally looking for a way to solve a particular problem. But there are so many particular problems that they cannot be packed into the confines of a single volume. A number of excellent, general and specialized reviews of chromatography are readily available, and every year summaries and reviews appear to which the Chemical Abstracts and corresponding library tools lead. The worker who knows nothing about chromatography can readily find methods to copy, and some specially chosen ones are given in this book. The specialist, who may have been using chromatography in a certain problem-area, is likely to know the literature of that area far better than any outsider. These considerations—together with the knowledge that no author can provide his readers with detailed instructions for all contingencies (since the essence of research is that new work poses new problems) and that detailed treatment of a large number of examples would be unprofitably repetitive—have set the pattern of this book. I have tried to show the principles of chromatography by the use of appropriate illustrations. I have tried to deal throughout with both the "how" and the "why" of chromatography, with the hope that readers will

be helped to enjoy solving their problems, and the experts will find here an interesting point of view. I have occasionally sacrificed popular usage for clarity, as in using "downward" and "upward" in place of "descending" and "ascending" in paper chromatography. Also, in the interests of clarity of presentation I have in a given treatment used the deductive or inductive approach, whichever seemed more felicitous. Here and there throughout the book I have introduced discussions of an epistemological kind where such seemed called for. I have tried to present the reader with a homogeneous treatment of the entire field of chromatography.

The plan of the book is as follows. In Chapters I and II important definitions are given, chromatography is placed in context in the field of separation methods, and the entire field of chromatography is classified in functional terms. Chapter III is introduced in order to survey the interactions between molecules upon which chromatographic separations are based. In my experience, a survey of this kind is welcome to workers who have not recently been in contact with theoretical chemistry. In any case, it defines phenomena which will be called upon throughout the book to explain observations. Chapter IV, on theory, provides the conceptual scaffold upon which explanations will later be hung. The next seven chapters deal with particular methods of chromatography. These are almost all presented according to the same general plan: the principle of the method is stated; apparatus and materials are discussed; actual manipulations are described; and wherever possible a careful conceptual analysis, followed by a discussion, is given of the factors that influence the separation of materials by that particular method. Chapter XII, which treats methods of recognizing and evaluating zones, is, in effect, a check-list of types of methods, since the number and variety of methods at present available are far too great even to be listed. The relations sought and found between **R** and $\mathbf{R_F}$ values and molecular structure in chromatographic separations are gathered in Chapter XIII to show the beginnings of what is becoming an important area in chromatographic research. The remaining two chapters attempt a heuristic approach to chromatography and its applications. Finally, I have gathered together in an Appendix, for the convenience of the reader but implying no endorsement on my part, lists of sources of chromatography equipment. The references have continually presented a problem. Very likely I have omitted to mention important work, and if so I should appreciate being told about it. My coverage of the literature has not been complete, as may be expected from the figures given in the first paragraph of this Preface, but I hope that in spite of this I have not committed any very serious omissions. Lastly, I may add that there is very little of the earlier volume, *Adsorption and Chromatography*, surviving in this book. This is a new book, not a revision.

I have written this book for everyone who would like to use chromatography, or who would like to improve his present methods or develop new methods. It is intended for all these users whether they are organic, inorganic, or biological chemists.

I have tried to acknowledge the sources of all figures and tables taken from the literature, and herewith thank their authors and publishers for permission to use them. I am especially indebted to those authors, and their publishers, who have permitted me to quote from their work. Extensive quotations were generously permitted by Dr. A. J. P. Martin and his colleagues, Drs. G. A. Howard, A. T. James, and R. L. M. Synge (Chapter IV, Sections V, X; Chapter V, Sections IV, VIII; Chapter VI, Section VI; Chapter XIII, Section V). Quotations were kindly permitted, also, by Drs. F. H. Pollard and J. F. W. McOmie (Chapter VI, Section VI); S. G. Thompson, B. G. Harvey, G. R. Choppin, and G. T. Seaborg (Chapter IX, Section X); Dr. D. F. Mowery, Jr. (Chapter IX, Section X); Drs. J. N. Balston and B. E. Talbot (Chapter VII, Section IV); and Drs. D. French and G. M. Wild (Chapter XIV, Section IV). Important information, with permission to publish it, was provided by Dr. Melvin Hatch of the Dow Chemical Company (Chapter IX, Section VII); by Dr. A. B. Littlewood (Chapter V, Section IV); and by Drs. G. J. Pierotti, C. H. Deal, E. L. Derr, P. E. Porter, F. H. Stross (Chapter XIII, Section VI) and J. J. vanDeemter (Chapter V, Section IV) of the Shell Development Company. I thank them for their assistance in making this book as advanced as possible.

The manuscript was read by Dr. A. F. S. A. Habeeb, Dr. W. J. Knox, Jr., and the editor, Dr. Arnold Weissberger, and I express here my thanks to them for their many valuable comments.

The manuscript was prepared with the assistance of my wife, and of Miss Teresa M. Carrano, who typed the tables, and Mr. George Teeber, who helped to check the bibliography. Their patient assistance and that of the editor and publishers of this series are acknowledged with pleasure.

Sterling Chemistry Laboratory
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April, 1957

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FUNDAMENTALS OF CHROMATOGRAPHY

CONTENTS

Introduction

I. HISTORICAL

Up to the early nineteen-forties, it could have been said that the history of chromatography was simple: Mikhael Tswett invented the method in the early nineteen-hundreds (947). It was known that fixed beds of charcoal had been used long before this to clarify solutions; and the works of Runge, Schoenbein, and Goppelsroeder were known, yet it was generally felt that such applications could not be called chromatographic. However, the situation was changed when in the early nineteen-forties more processes, some newly invented, others not, began to be spoken of as chromatographic.

It is obvious that if the definition of a method is changed, then its history will have to be revised. As long as chromatography was Tswett adsorption analysis, the history was fairly definite, though as Zechmeister pointed out "when the time is opportune for the clear formulation of a new principle, several minds may simultaneously have the information on which to base it," and the work of Day and his colleagues might have developed into systematic chromatography. That Tswett knew of Goppelsroeder's experiments on capillary analysis is shown by his mention of this work in his early papers. However, when chromatography comprises frontal analysis (which does not involve an explicit development step) and methods like ion exchange, then the history of chromatography can trace the origins of chromatography very far back indeed.

This being the situation, as we see it (and we must also add that the history of chromatography is largely irrelevant to the actual practice of chromatography), we will not attempt an historical survey here. The reader who wishes to pursue the subject is referred to the authors already cited and to the writings of Zechmeister, Weil and Williams, and others (973,974,977,1006,1030). There he will find also an account of an important scientific phenomenon, referred to by Henri Focillon in another connection as "the innumerable mistakes that lurk in the shadow of success."

II. DEFINITION OF CHROMATOGRAPHY

There have been many definitions of chromatography. The one that seems most satisfactory and that will be used to limit the contents of

this book may be derived as follows. Chromatography is primarily a *separation* process which is used for the separation of *essentially molecular mixtures.* Thus it is incorrect in this view to think of chromatography as a "sifting" or "filtration" process, since these terms apply strictly to macroscopic—or microscopic—but not to molecular-size particles. Further, chromatography depends on the redistribution of the molecules of the mixture *between two or more phases,* in this respect bearing a relation to distillation, liquid–liquid extraction, and such separation processes, as is further discussed below. But this excludes such molecular separation processes as mass spectrometry or ultracentrifugation. More specifically, the distribution that is chromatographic involves *one phase that is essentially two-dimensional,* that is, a *thin phase.* Here, still, there is some lack of exclusiveness, but in general the kind of distribution that occurs in chromatography is in some aspect a distribution between a bulk phase and an interfacial phase or a thin film of liquid. This rules out of the chromatographic category such bulk-phase distributions as ordinary liquid–liquid or absorption distributions, and vapor–liquid distribution (distillations). Finally, simple batchwise adsorption is excluded by the requirement that for the distribution to be a chromatographic one, the phases must be contacted at least countercurrently, and most specifically in a differential countercurrent manner. Thus chromatography is epitomized as *a separation process applicable to essentially molecular mixtures which relies on distribution of the mixture between an essentially two-dimensional, or thin, phase and one or more bulk phases which are brought into contact in a differential countercurrent manner.*

But scientific definitions being what they are it would not be profitable to adhere undeviatingly to the above definition. For one might be hard put to it to state to the satisfaction of everyone how thick a surface has to become before it will be recognized as bulk phase. Thus in some aspects "partition chromatography" shades off into a liquid–liquid distribution of the ordinary packed column type; and gas–liquid partition chromatography into packed column carrier distillation. In other aspects, as when a zone on an adsorbent accumulates into multilayers of oriented molecules it approaches crystallization phenomena, and so on. We will nevertheless use the above definition, which quite fairly epitomizes the present chromatographic process, as our definition here. Nothing that has been said excludes the combination of chromatography with any other separation process, or with part of another process.

III. CONTEXT OF CHROMATOGRAPHY: THE FIELD OF ANALYSIS

In making the definition of chromatography as clear as possible, it is essential to place this method in its proper context in the field of analysis. Analysis is the resolution of a complex whole into its parts. There are in general three kinds of analysis: qualitative, quantitative, and relational or structural. In each of these the analytical procedure may or may not involve a separation process, and the three kinds of analysis may be carried out in sequence, or in combination, or in any order, though usually the first two precede the third. Analytical schemes are summarized in Table I-1. This is a formal scheme devised to give a general

TABLE I-1
Summary of Analytical Schemes

- Bulk level
  - Sorting and sifting; filtering. Mechanical separation of phases
- Molecular level
  - Physical analysis
    - with no physical separation
    - Physical analysis with physical separation
      - Distribution between phases
        - See Table I-2
      - Thermal analysis, e.g., thermal diffusion
      - Gravitational analysis, e.g., centrifugal analysis
      - Electrophoretic analysis
      - Electromagnetic analysis
      - Mass spectrographic analysis (belongs also under submolecular level)
  - Chemical analysis with or without separation
- Submolecular level
  - Chemical analysis with separation. Bonds are broken
  - Chemical analysis with no separation: optical, electrical, magnetic methods

picture of an area. It will be impossible to make *very sharp* distinctions between categories in many cases. For example, ion exchange is a distribution between phases which can be classified under physical methods as a special case of adsorption. However, ionic bonds are broken, and ions are submolecular species, so it can also be placed under chemical analysis with separation. Because we cannot go very far into episte-

mological considerations here, we shall adopt Table I-1 as an approximation which will help to place chromatography in context among analytical schemes (171a).

In chromatographic analysis we are concerned with the analysis of molecular mixtures. In general such analyses, when they involve *separation*, as does chromatography, *rely upon an attempt to convert the molecular level separation into a bulk level separation, which, because it involves bulk, macromolecular, phases, is relatively easy to carry out and requires merely a mechanical operation* (162,168). The analytical methods of this kind are summarized in Table I-2. In effect, the mixture

TABLE I-2
Cross-Table of Phase-Pair Distributions and the Methods That They Generate

	Bulk phases		
	Gas or vapor (G)	Liquid (L)	Solid (S)
Bulk Phases			
Gas or vapor (G)	G–G[a] (Atmolysis; gas diffusion)		
Liquid (L)	G–L (Distillation; absorption)	L–L (Solvent extraction; absorption)	
Solid (S)	G–S (Sublimation)	L–S (Crystallization)	S–S (Enfleurage)
Thin Phases			
Liquid film or "mobile" interfacial film (M)	G–M (Gas partition chromatography) (GPC)	L–M (Liquid-liquid partition chromatography; foam and emulsion chromatographies)	
"Immobile" interfacial film at the surface of a solid adsorbent (I)	G–I (Gas adsorption chromatography) (GAC)	L–I (Conventional adsorption chromatography, or Tswett adsorption analysis; ion exchange chromatography)	

[a] In this case an artificial interface, a membrane, or barrier must be put between the phases.

is placed in an environment (two phases in contact) in which a redistribution of the components of the mixture occurs between two phases that can be separated mechanically, yielding mixtures of different and more desirable composition.

Inspecting the cross-table, I-2, we find that the separation processes which depend upon the distribution of a mixture between two phases are given different names depending upon the kinds of phases involved. Adsorption is the term used when one of the phases is essentially a surface phase. When it is doubtful whether the phase is a surface one or is thick

enough to be called a bulk phase or when both possibilities are under consideration, the more noncommital term "sorption" is used (576).

There are two kinds of procedures by which any pair of phases may be contacted: (1) the batchwise and the cascade (or multiplied batchwise) procedures, and (2) the differential countercurrent (Chapter IV). Examples of some of these are listed in Table I-3. The first two of these

TABLE I-3
Examples of Methods of Operation by Which the Various Indicated Phase Pairs Are Utilized in Separation Processes

Separation process	Distillation	Solvent-solvent extraction	Adsorption	Crystallization
Phase pairs utilized	(G-L)	(L-L)	(L-I)	(L-S)
Batchwise	Distillation of a volatile substance from essentially nonvolatile solvent	Simple liquid-liquid extraction	Simple decolorization	Simple crystallization
Cascade (multistage)	Bubble-cap tower operation	Systematic repeated extraction	Systematic repeated adsorption	Systematic recrystallization
Differential countercurrent	Packed tower operation	Packed tower extraction	Chromatography, Hypersorption, Rotosorption	Countercurrent crystallization

may be carried out as equilibrium distributions. On the other hand, a condition of overall disequilibrium may be utilized, as in the differential countercurrent procedures.

It should be stated, for the sake of completeness, that batch and cascade processes may be carried out at disequilibrium if an advantage is thereby secured (for example, if the components of a mixture distribute at very different rates) but at least they can be equilibrium processes. This is one of the outstanding virtues of the Craig countercurrent (cascade-type) distribution method ("CCD" method). It allows accurate calculation of every step in the process.

IV. OTHER DEFINITIONS OF CHROMATOGRAPHY

The definition of chromatography and this rather formalized presentation of the relation of chromatography to other analytical methods is designed to help clear the air of partial and otherwise inadequate analyses of the nature of chromatography. It will be completed by the classification

of chromatographic methods in the following chapter. The definition may have to be changed when radically new methods of chromatography are invented, or when old methods, presently excluded from the charmed circle of chromatographies, are discovered to be really, after all, chromatographic. No prescience is claimed for the definition.

It is, indeed, because of these considerations that a less formal definition of chromatography such as that of Martin (612) may have an advantage. He wrote, "The essence of the chromatogram is the uniform percolation of a fluid through a column of more or less finely divided substance, which selectively retards, by whatever means, certain components of the fluid." However, such a definition is by no means inclusive, nor specific for chromatography. On the other hand, Williams and Weil (1007) propose the definition: "By chromatography is meant those processes which allow the resolution of mixtures by effecting separation of some or all of their components in concentration-zones on or in phases different from those in which they are originally present, irrespective of the nature of the force or forces causing the substances to move from one phase to another." This definition is so inclusive as to be useless, for it would include distillation under reflux, the Craig countercurrent distribution, zone melting, and other processes that are not accepted as chromatographic

CHAPTER II

The Nature of Chromatography

I. ILLUSTRATION OF ADSORPTION CHROMATOGRAPHY

The definition of chromatography given in the preceding chapter embraces all kinds of chromatography. It may be illustrated with an example of the separation of two dyestuffs, Sudan red and Sudan yellow, on alumina. The alumina adsorbent is sufficiently active to adsorb both dyestuffs, and it holds the Sudan red more firmly than the Sudan yellow. The procedure as described by Brockmann and Schodder (118), is as follows. Alumina is filled into a chromatographic tube of 1.5 cm. internal diameter to a height of 5 cm. This gives a *column* or deep *bed* of adsorbent A, Fig. II-1,*a*. A disk of filter paper is placed on top of the column to help prevent stirring of the adsorbent when liquid is introduced. The mixture of dyestuffs comprising 2 mg. of each in 2 ml. pure benzene and 8 ml. petroleum ether is then *applied* carefully to the top of the column, without disturbing the adsorbent (Fig. II-1,*b*). As this passes into the adsorbent, a

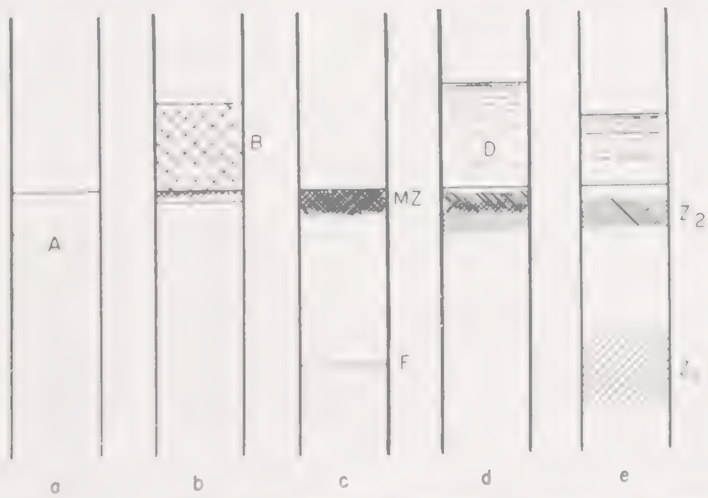


Fig. II-1. Chromatography of a binary mixture. *A*, column of adsorbent in the chromatography tube. *B*, solution of mixture to be separated. *MZ*, mixed zone, applied to the column. *F*, front of empty solvent. *D*, developer, applied to the column. Z_1, Z_2, zones of separated components. To the packed column (*a*) the solution to be analyzed is applied (*b*). As it passes into the bed of adsorbent the components are retarded to form a mixed zone, while the solvent (also to some extent adsorbed) runs ahead (*c*). Developer is then applied (*d*), and as the development occurs the zones of separated material draw apart to produce the developed chromatogram (*e*).

mixed zone, or *initial zone*, of the adsorbed dyestuffs forms at the top of the column while the front F of the empty solvent moves ahead through the bed, wetting it in passage (Fig. II-1,*c*). When the solution has just all passed into the column, a *developer, D*, composed of benzene and petroleum ether in the ratio, 4:1 by volume is applied to the column (Fig. II-1,*d*). As this flows in, the initial mixed zone broadens and differentiates into two zones, an upper of Sudan red (Z_2), and a lower of Sudan yellow (Z_1), Fig. II-1,*e*. These zones draw apart, and after 20 ml. of developer has passed, at a flow rate of 20 to 30 drops per minute, the two are well separated. The more strongly adsorbed Sudan red forms a zone about 1 cm. deep near the top of the column, and the less strongly held Sudan yellow forms a broader zone below it.

In this example, as in most kinds of chromatography, distribution of the substances to be separated occurs between a bulk phase which passes over the dispersed "two-dimensional" phase. The bulk phase, then, is the *mobile phase*, whereas the thin phase at the surface of the particles of the bed is the *stationary phase*. In effect, one phase moves against the other in a differential countercurrent manner.

The process, which has been described in terms of operations and observations, may be described in terms of inferred mechanism, using as the key phenomena the facts that one phase moves over the other and that the solutes (the dyestuffs) distribute themselves between both phases. This distribution occurs because the dyestuff molecules interact with the molecules of the solvent as well as with the surface molecules of the adsorbent. The nature of the interactions that may operate in chromatography is left for the following chapter.

When a solution of adsorbable substances (*adsorptives*) is brought into contact with a quantity of an adsorbent, molecules of adsorptive pass out of the solution into the interfacial region, where they are retained for a longer or shorter period of time depending on the strength of the adsorption. As the concentration of the adsorptive builds up in the interface the solution is depleted of adsorptive. But a reverse process, the escape of adsorptive from the interface into solution (*desorption*) also is occurring, and eventually an equilibrium state may be reached when both reactions balance each other. The resulting equilibrium distribution system may be represented thus:

$$\text{rate of desorption} = \text{rate of adsorption}$$

This statement can be applied to chromatography by restating the situation thus:

Passage of substance into mobile phase opposes
passage of substances into stationary phase

Returning to the illustration of Fig. II-1, the adsorbent *A* may be considered a support for the stationary phase, which will be the interfacial film formed *by the adsorbed solvent, as well as by the dyestuffs*. All these adsorptives will compete for the surface during the chromatography. When the mixture is first applied to the column, the first small increment of solution comes into contact with empty absorbent. Solvent and solutes are adsorbed, as would be expected from the distribution relationship when there is no possibility for the reverse reaction to occur (because the bed is empty). The solvent is, however, poorly adsorbed compared with the solutes. This means that the dyestuffs are preferentially adsorbed, and so displace solvent from the interface. Since it is a small factor, the adsorption of solvent will be ignored.

If this first increment, or thin layer, of mobile phase were to remain in contact with the same thin layer of stationary phase, equilibrium would eventually be established. (This would be a batchwise type of distribution process.) But the second key factor in chromatography, the movement of the mobile phase, prevents this, for this thin initial layer moves on down the empty bed, continually losing adsorptive, until within a very short distance it has been completely emptied of adsorptive, and thenceforth moves on down the adsorbent, wetting it. (Oftentimes one can feel the heat of wetting at the front of the solvent.)

The next increment, or thin layer, of solution passes at first over an interface that already contains some adsorptive which it received from the first thin layer. But if the stationary phase is not saturated with respect to solutes, then more will be adsorbed, according to the relation:

$$\text{substances in mobile phase} \xrightarrow[\leftarrow]{} \text{substances in stationary phase (phase is not saturated)}$$

(The arrows imply a dynamic situation: the rate of adsorption being greater than that of desorption.) This process will deplete this increment of solution. It will flow onward, eventually losing all its solutes as the first increment did.

As each increment of solution flows over the first thin layer of adsorbent the amount of solutes adsorbed increases until, if the volume of the mixed solution is sufficient, and/or the rate of adsorption is rapid enough, the phase comes into a steady-state distribution relation with the components of the mobile phase:

$$\text{substances in mobile phase} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{substances in stationary phase}$$

and k_1 equals k_2, where these are the rates of the two halves of the distribution reaction at equilibrium. Thus by this process of accumulation of

adsorbed material the initial mixed zone is built up. It is not a necessary condition for this stage in the chromatography that the steady state just described be reached within the zone. It is only necessary that the substance(s) in the mobile phase pass into the stationary phase, and that the mobile phase move over, along or through, the stationary phase. These are the conditions for zone formation.

When all the mixture has passed into the bed, or column, of adsorbent (Fig. II-1,*c*) the mixed zone contains both adsorptives *on* the adsorbent, and also *in* solution in the interstices between the particles of adsorbent. Moreover, since Sudan red is more strongly adsorbed than the yellow, it will have displaced some yellow near the top of the mixed zone, whereas the lower edge of this mixed zone will be enriched in Sudan yellow.

Developer is now applied to the column (Fig. II-1,*d*). In this illustration the developer contains a higher ratio of benzene to petroleum ether than did the original solvent (4:1 as against 1:4). The developer therefore dissolves these aromatic dyestuffs better than the initial solvent and competes more favorably for them with the adsorbent surface.

As the "empty" developer comes into contact with the top of the mixed zone, adsorptives are *desorbed*, since there is at first no reverse component in the distribution:

substances in mobile phase ⟵—— substances in stationary phase
(None—the developer is "empty")

The moving developer, as it passes over the zone, continues to pick up adsorptives (possibly until a steady state is reached), but it picks up relatively more of the less strongly held Sudan yellow than of the red, which also continually displaces yellow on the adsorbent to the extent that it can. When the developer, loaded with adsorptives in its passage over the zone, reaches the lower or *leading edge* of the zone, it then loses its burden of adsorptive to the empty adsorbent as was described for the formation of the mixed zone. Thus the developer liquid, as Tswett put it, starts with zero concentration of solute, picks up substance in its passage through the zone until a maximum concentration is reached, then returns to zero concentration after depositing the substance at the lower edge of the zone. This behavior causes the zone to migrate in the direction of flow of the liquid. It demonstrates very beautifully the operation of Le Chatelier's principle. The rate at which the zone migrates is mediated by the rate of flow of the liquid as well as by the strength of adsorption of the substance involved. The more strongly adsorbed substance moves less rapidly than the one less strongly held, other conditions being equal, since it is less completely desorbed by the developer in its passage through the zone. This indeed is the basis of the sorting or

separation process. For if the substances in the mixture are adsorbed with different degrees of tenacity, then their zones will move at different rates under the influence of the flowing developer liquid, and hence if other conditions are favorable the zones draw away from each other and become completely segregated. At this stage the chromatogram is said to have been developed or to have become developed (Fig. II-1,ϵ).

This illustration shows the essentials of any good chromatogram: the zones are compact, they are quite sharply defined, they are well separated, and the process does not require an excessive amount of time or material. The resulting chromatogram can be described by reporting the depths of the zones and their positions relative to the top of the column, for the particular system used. A more fundamental method of description is given in terms of the **R** value of each zone. This is the ratio of the velocity of movement of the front edge of the zone to the movement of the developer liquid meniscus in the empty part of the tube above the column of adsorbent:

$$\mathbf{R} = \frac{\text{velocity of movement of the front edge of the zone (mm./min.)}}{\text{velocity of movement of developer in tube above the column of adsorbent (mm./min.)}}$$

R is a measure of the retardation of the zone. Of the two substances illustrated in Fig. II-1, the Sudan red (Z_2) will have a smaller **R** value than the Sudan yellow (Z_1). The smaller **R** value always means that the molecules of that substance distribute into the stationary phase to a relatively greater extent than do the molecules of the substance the zone of which has a higher **R** value. This is because it is only when the molecules are in the mobile phase that they move down the column, thus giving the zone its **R** value.

II. ILLUSTRATION OF PAPER PARTITION CHROMATOGRAPHY

Another example will be given, in less detail, to illustrate these same principles applied to paper chromatography. This example is composed from the first detailed paper published on this subject (191).

A strip of Whatman No. 1 filter paper 1.5 cm. wide and 20 or more cm. in length is marked lightly with a pencil line 5 cm. from one end. At a marked spot in the middle of this line there is applied the mixed zone of the amino acids which are to be separated. The mixture, containing glycine, alanine, valine, and leucine, about 5 to 15 micrograms (μg.) of each acid in 2 to 4 microliters (μl.) of total solution, is delivered from a capillary pipette onto the paper as a small round spot, and the solvent is

allowed to evaporate (Fig. II-2,*a*). The paper, which deputizes for the column of adsorbent in the first example, is hung in a chamber, with the upper end held in a trough. In the bottom of the chamber there is a dish to hold liquid (Fig. II-2,*b*).

The developer is made by shaking together liquefied phenol and water in about equal portions in a separation funnel for several minutes, to saturate each with the other. The phases are then allowed to separate cleanly, and the upper, aqueous phase is drawn off into the dish which is

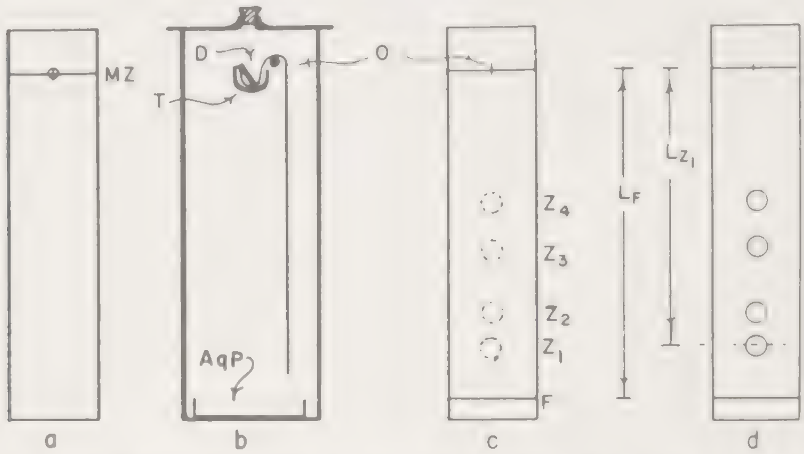


Fig. II-2. Paper chromatography of four amino acids. *MZ*, mixed zone. *T*, trough. *AqP*, aqueous phase in a wide-diameter dish. *D*, developer, organic phase. *O*, center of origin of mixed zone. *F*, front of developer liquid. Z_1, Z_2, Z_3, Z_4, zones of amino acids. L_F, distance moved by front beyond point of origin of mixed zone. This distance is measured in a straight line from *O* to the line of *F* perpendicular to the line through *O*, unless there is evidence that the zones swerve to one side, when the measurement becomes uncertain. L_{Zi}, distance moved by center of zone *i*. $\mathbf{R_F}$ is defined as L_{Zi}/L_F.

placed at the bottom of the chamber (Fig. II-2,*b*, *AqP*). This liquid is introduced to saturate the air in the chamber with water and phenol in the ratio that will be in equilibrium with the developer. The paper containing the mixed zone is allowed to hang in the chamber for a while so that it may sorb the reagents and become in this process *conditioned* to them.

Now the developer which is the lower, phenolic phase is introduced into the trough through a hole in the lid of the chamber, and this is resealed. The developer moves by capillarity into the paper and, aided by gravity, passes down over the mixed zone. The resulting development proceeds in principle as with the column of the first example. Here, the bulk, mobile phase is phenol containing water, and the thin stationary phase is water with some phenol in it sorbed to the cellulose support. In the first example

the outer limit of the column is set by the glass walls of the chromatography tube; here it is set by the surface forces of the developer. Thus this example differs from the first not only in apparatus, but also in that it involves essentially a liquid–liquid distribution, while the first relied on a liquid–solid interface distribution. *The principle of the separation is the same in both cases.* The conditions for zone movement and differentiation are in principle the same.

The amino acids of this example are colorless, and so in order to reveal the position of their zones, a color reaction with ninhydrin is used. After the front of the developer has moved almost to the lower edge of the paper, the chamber is opened, the paper removed, and the position of this front marked immediately. The paper is then allowed to dry. It is sprayed with a solution of ninhydrin (0.1% in *n*-butyl alcohol) and heated for a short time to accelerate the reaction with the amino acids.

The amino acid zones appear as purple or red-purple spots (Fig. II-2*d*). The chromatogram is described (and the zones are characterized) in terms of an R_F value which is the analog of the R value on the column. The R_F measures the velocity of movement of the zone relative to that of the developer *front*. (It would be difficult to measure an R value with paper.) The actual measurement is made at the end of the described process by measuring the distances from the starting line—the center of the initial mixed zone—to the developer front and to the center of density of each zone.

$$R_F = \frac{\text{distance from starting line to center of zone}}{\text{distance from starting line to solvent front}}$$

From this definition it is apparent that the substances that are more retarded will show smaller R_F values than those that move faster. Analogously to the first example, for the principle is the same, substances with smaller R_F values are those that distribute to a relatively greater extent into the stationary phase. It is only when molecules are in the mobile phase that they move effectively down the paper.

III. KINDS OF CHROMATOGRAPHY

The illustration and discussion of these two chromatographic processes, one adsorption, the other liquid–liquid, given in the previous section apply in essentials to all the various kinds of chromatography.

The differences between the various kinds of chromatography lie at two main levels, differences in the kinds of distribution systems used, particularly in terms of the phases employed (see Table I-2), and differences in the ways the phases are contracted or manipulated.

There are four major kinds of chromatography: classical, or adsorption chromatography; fluid partition chromatography; ion exchange; and foam and emulsion chromatography. The position of electron exchange is not yet clear. Some of these may be subdivided. They are each treated later in separate chapters, and are only briefly described here in terms of the phases employed.

Each of these kinds of chromatographic systems comprises a group of methods which differ from each other in the ways that the phases are contacted, or manipulated. These methods have been given felicitous names by Tiselius, who invented one of them (927,927a,929,930). They are frontal analysis, development analysis, and displacement analysis. These are not mutually exclusive methods, and further, they may be subdivided into distinguishable processes which bear their own special names. However, it is useful to fasten upon this scheme of classification as being simple and based on fundamentals. Elaboration of the scheme will follow as each system is discussed. In principle, each kind of distribution system can be the subject of every sort of manipulation scheme.

Tables II-1 and II-2 contain lists of the chromatographic systems and methods.

TABLE II-1
Chromatographic Systems

Name	Mobile phase	Stationary phase
Classical, or Tswett, or adsorption chromatography	Bulk fluid	Interfacial phase at the surface of an adsorbent
Ion exchange chromatography	Bulk fluid	Ion pairs and solvation region at the surface of the resinous or inorganic backbone of the resinous or zeolitic support
Partition chromatography	Bulk fluid	Liquid or gel film that takes part in the distribution and is sorbed to a support
Foam and emulsion separation	Bulk fluid	Interfacial film on bubbles or droplets

TABLE II-2
Methods of Contacting or Manipulating Phases

Name	Characterization
Frontal analysis	Small bed, excess of mixture; no developer
Development analysis, elution analysis	Large column, small amount of mixture; developer used
Displacement analysis, carrier displacement	Large column, small amount of mixture; minimum of developer-displacer used

Classical or **adsorption chromatography** relies on the distribution of the components of the mixture to be separated between a bulk fluid and an essentially nonmobile interface; that is, upon an adsorptive distribution between a fluid mixture and the surface of a solid adsorbent.

An adsorption process is involved in **ion exchange chromatography** also, but here the adsorptive is ionic, and adsorption takes place, as a competitive process, at fixed charge sites on the "surface" of the exchanger. In commercial ion exchangers the number of available sites for exchange is relatively large, and the exchanger may be looked upon as a solid polyelectrolyte, which interacts then, upon its "surface," with ions in the contiguous phase. Thus the distribution is a "surface" versus bulk liquid distribution. The difference in behavior between ion exchange adsorbents and other adsorbents is not necessarily a sharp one. The distinction is made chiefly on the basis of the mechanisms of the predominating reactions. Further, ion exchange *resins* may react as sorbents for nonionized, and some other, substances.

In **partition chromatography** there are in the main three systems: gas partition, liquid partition employing fixed beds, and paper chromatography. In each case the distribution takes place between an essentially stationary sorbed "liquid" phase, and a mobile fluid in intimate contact with it. In gas partition chromatography a mobile gaseous phase passes over an essentially stationary liquid phase sorbed to a support which may take part also in the distribution process, but which preferably does not. Liquid partition chromatography utilizes the stationary phase sorbed to a support with a liquid mobile phase in contact with it. In paper chromatography the support is paper, or treated paper or strips or sheets of other materials such as glass fibers.

Foam and emulsion chromatography, or "fractionation," utilizes a distribution between a solution and a "*mobile*" *interface*. Bubbles of gas or droplets of liquid are introduced into the solution and rise or fall through it, thus providing the interface. It is "mobile" because it is formed between two fluids. This distinguishes the interface from that which is formed in the region of contact of a fluid with a solid, which is called an "immobile" interface.

There are systems in use in which chromatography is combined with some other separation method. One of these is electromigration chromatography. Here an electromigration is imposed upon a chromatographic process. This method is not discussed in this book.

IV. METHODS OF CONTACTING THE PHASES

The various methods of contacting or manipulating the phases are in principle applicable to all these chromatographic systems. The methods

themselves are described for each system in the appropriate chapter. The following is a quite general survey.

Frontal analysis is a method of chromatography in which the mixture is passed into a bed (or strip) of sorbent, without development. The components of the mixture are sorbed to different extents (if they are separable by this sorbent). The most strongly sorbed is retarded most

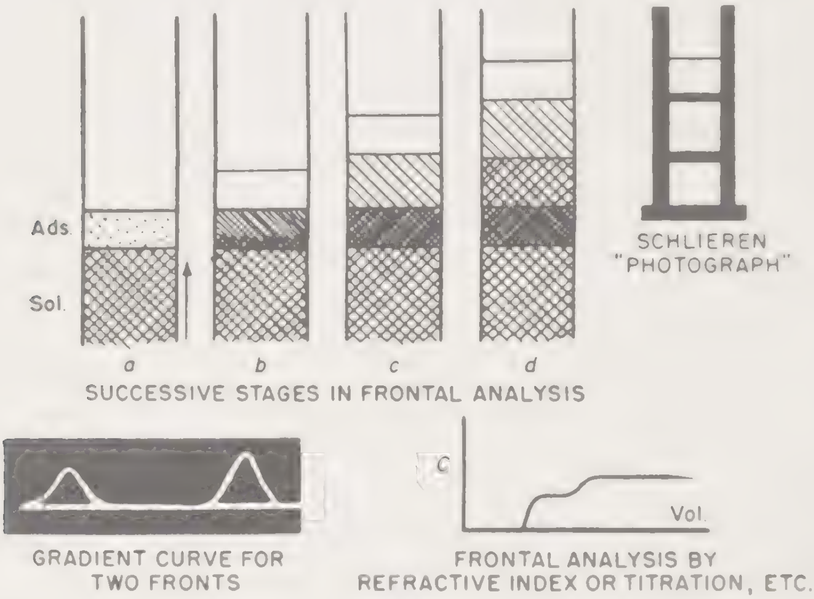


Fig. II-3. Successive stages in the frontal analysis of a binary mixture. (*a*) The solution of substance *A* and *B* is about to enter the empty adsorbent, Ads.; (*b*) the adsorbent is saturated with substances *A*, and partly with *B*; (*c*) the front of *A* has appeared with empty solvent (retention volume of *A*) above it, and the adsorbent is shown just saturated with *A* and *B*; the front of *A* + *B* is about to appear; (*d*) the fronts of *A* and *A* + *B* have appeared. The retention volume of *B* is the entire volume above the front of *A* + *B*. A schlieren photograph is shown diagrammatically at the right for these fronts. Below are gradient curves showing regions at two fronts where refractive index *changes* sharply. A frontal analysis diagram is shown at the lower right, where *C* is concentration determined by any means. The distance up to the first "step" is a measure of the retention volume for *A*, and up to the second step for *B*. (After Tiselius (930).)

in its passage through the bed. The less strongly sorbed components are retarded to a lesser degree. If the bed is a shallow one, and an excess of mixture is used, and if all the components are to some extent sorbed, first the empty solvent appears ("breaks through"), then the least strongly sorbed component, then the next most strongly sorbed component mixed with the first, and so on (Fig. II-3). The method, subject to its limitations, tells the number of components in the mixture. If the bed of sorbent is

deep with respect to the amount of mixture, as is often the case in capillary analysis when the "bed" is a strip of paper dipping into the mixture, the pattern of retardation of different species appears in the bed.

Development and elution analysis always use beds of sorbent that are deep relative to the amount of mixture to be separated. The mixture is sorbed as a zone near one end of the bed (top or bottom), or at the center if a disk of sorbent is used, and is then developed by passing over it a liquid which desorbs the components to differing extents. The more readily desorbed components pass more easily into the developer and so are carried along relatively more efficiently by the moving developer than the more strongly sorbed components. Under favorable conditions zones of the components are separated from each other along the bed of sorbent in the direction of movement of the developer (the mobile phase) and in the order of strength of sorption—the more strongly sorbed substances being retarded most, so that zones of these substances lie closer to the place of application of the mixture (Figs. II-1 and II-2).

Displacement analysis is a form of development in which a developer is used which displaces all the components of the initial mixed zone from the sorbent. As this developer passes into the bed of sorbent it pushes the mixture ahead of it (acting as a sort of molecular piston). If the components of the mixture are chemically (and thus physically) alike in their behavior, differing only in extent of sorption but not essentially in sorption interactions, that is, if the components of the mixture are related as homologs would be in their behavior, then the most strongly sorbed will displace ahead of its zone, the next less strongly sorbed, and so on, so that after a steady state has been established the zones move one ahead of the other, all pushed by the displacer. Very weakly retarded substances may move ahead of this array of zones. The separated zones may then be collected in order as they emerge from the column (Fig. II-4).

These three are the major ways of contacting the phases in the various types of chromatography. They may be further subdivided to distinguish gradient elution analysis, carrier displacement analysis, and so on. But these divisions are not so fundamental, and will all be treated in detail in the appropriate chapters.

Very many changes can be rung on these themes. The mobile phase may be made to move upward or downward or in any other direction such as horizontally or radially through the stationary phase mass. Or the quondam stationary phase may be passed through the bulk phase by sifting it if it is a solid adsorbent, or spraying it if it is a liquid, into a column of the bulk phase. The movement of the mobile phase may be produced under the pull of gravity; it may be aided by suction applied

to the stationary phase, or it may be forced in under pressure. Centrifugation, capillary forces, or other agencies may be utilized to move the one phase over the other. All cases so far known seem in their essentials to fall within the rubric of Tables II-1 and II-2.

This rather formal analysis and presentation of the kinds of chroma-

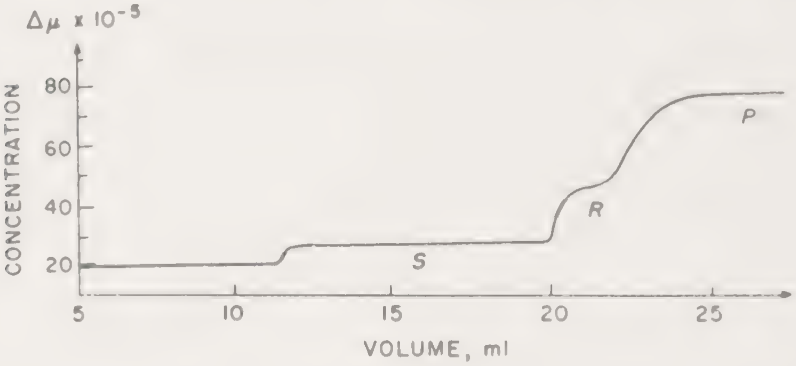


Fig. II-4. Displacement analysis. The concentration (in terms of refractive index) is plotted against the volume passing the foot of the column. The displacer, *P*, is 0.25% phenol, which displaces raffinose, *R*, which displaces sucrose, *S*. These two substances are segregated into separate zones. Under standard conditions the height of the zone characterizes the substance; the length of the zone measures the amount present. (After Tiselius (930).)

tography is designed to complement the analysis in the previous chapter and to give a broad view over the entire field so that connections can be seen between all the different kinds. It is a classification primarily for the purposes of action, rather than for the filing case. Yet it has its uses too in organizing the ever-growing number of publications on chromatography.

THE MOLECULAR INTERACTIONS ON WHICH CHROMATOGRAPHIC SEPARATIONS REST

I. INTRODUCTION AND DEFINITION OF INTERACTION

As a mobile phase moves over a stationary phase in a chromatographic system, there is a constant interchange of the molecular components through the region of contact of the two phases. This interchange is governed by interactions between the molecules of the system. Some of these molecular interactions cause the stationary phase to adhere to the supporting substrate; they or others give the stationary phase its thickness and integrity. Some interactions are responsible for the velocity of flow of the mobile phase through their effect on its viscosity, density, and interfacial tension against the stationary phase. In paper chromatography they influence the thickness of the mobile phase by controlling its surface tension (since there is no tube wall to give an outer boundary to the chromatogram, as there is in column chromatography). If a mixed zone is present in the chromatographic system, there are interactions that control the relative extent to which the components go into the stationary and mobile phases. Zone molecules that preferentially enter the stationary phase because of molecular interactions of an associative kind with the molecules of that phase, or with the substrate, are retarded. Conversely, zone molecules that preferentially enter the mobile phase move along the column more rapidly. The degree of retardation is influenced by the composition of the mobile phase in that these molecules also compete, to some extent, for the stationary phase. All these competing interactions that make up the *system* are focused in the **R** or $\mathbf{R}_f$ values of the zones. It is the relative magnitudes of these values that the chromatographer wishes to explain and predict, and thus to control.

By interaction we mean physical attraction or repulsion between molecules or ions. These interactions are reversible. They vary from the rather weak to the rather strong and may approach in strength (magnitude of energy released) weak chemical reactions. Thus an ion–ion interaction is usually considered a chemical bonding reaction whereas an ion–dipole interaction is in many cases considered physical. There can be set up, in fact, a gamut of strengths of interactions: the lesser ones are physical interactions, or associations; the stronger ones are chemical

reactions, or bondings, and the division between the two is set at some convenient place in the scale.

It is the physical interactions that are of most interest in chromatography, for they control at the molecular level the behavior of bulk phases. But the physical interactions of molecules reflect the atomic composition and the constitution of the individual molecules as they are fixed by the chemical bonding forces. We therefore begin with a brief discussion of the latter.

II. CHEMICAL BONDS

To neglect the metallic bond, the chief classes of chemical bonds are the covalent, the ionic, and the coordinate. The **covalent bond** is formed when the two bonded atoms share a pair of electrons (subject always, of course, to the Pauli exclusion principle). According to simple valence theory (the molecular orbital approach yields the same results, but with more precision and detail), the shared electrons may be considered to spend a part of the time under the influence of each nucleus, thus giving rise to a fluctuating dipole, but to spend most of the time more fully under the influence of both nuclei. Since this is a sharing process, the pure covalent bond would normally occur only between like atoms because each would exhibit the same affinity as the other for the bond electrons. Such a bond would normally be *directed in space between the two bonded atoms.* The bonded atoms *would be mutually dependent in their electronic structures*, and the bond would exhibit *no overall permanent electrical assymmetry.* Such pure or nearly pure covalent bonds are found in paraffin hydrocarbons, and in alkyl groups, between C and C, and C and H.

The **ionic bond** is formed between two atoms or groups with opposite charges, as between RSO_3^- and Na^+ to form $RSO_3^-Na^+$ (where R is an aliphatic or aromatic residue). Here the energy required to remove an electron from the valence orbit of sodium is sufficiently less than that released when the last orbit of the oxygen is filled by the electron that the electron is *transferred*, rather than being shared. (However the ions were actually formed, this is the net effect.) The bond is due to electrostatic interaction. Its strength varies inversely as the square of the distance between the centers of charge. In distinction to the covalent bond, the ionic bond is normally *not directed in space.* The ions exert electrostatic effects in all directions out from their "centers," except as R groups shield certain regions. Thus a single ion may surround itself with many ions of opposite charge. Also, the bonded ions are not mutually dependent in their valence electronic structures.

A *majority of chemical bonds* fall between these two types: they are neither pure covalent nor pure ionic. The attraction that one of the bonded atoms has for the bond electrons is greater than that of the other, thus precluding pure sharing, yet it is not sufficiently strong to lead to actual transfer. The result is an unequal sharing: the probability of finding the electrons in the neighborhood of one of the atoms is greater than that of finding them nearer the other. This results in an electrically unsymmetrical bond—a dipolar or "polar" bond.

There are several ways to designate **polar bonds.** One frequently used symbol indicates the relative positiveness and negativeness of the bonded atoms thus:

$$\overset{\delta+}{\mathrm{C}}—\overset{\delta-}{\mathrm{Cl}}$$

In another convention the bond would be indicated $\overset{+\longrightarrow}{\mathrm{C—Cl}}$. These symbols indicate the presence of a dipole and show which end is negative and which is positive. The bond dipole can be measured in terms of a bond dipole moment.

The extent of the polarity difference between any two atoms and the direction of the resulting dipole can be judged from the positions of the atoms in the Periodic Classification Table. Metals tend to form ionic or strongly polar bonds with nonmetals. The tendency to take on electrons to fill out electron shells increases towards the right in any period, and upward in any group in the Table.

The importance of polar bonds lies in the fact that *whereas they are to some extent directed in space between the bonded atoms, they also exhibit a nondirected interacting ability.* The magnitude of this extra interacting ability depends on the magnitude of the bond dipole. It is possible on the basis of the Periodic Classification thus to judge in a general way the relative magnitudes of bond dipoles (for example, usually the C—N dipole will be smaller than C—O), but an analysis of the factors that contribute to the bond dipole indicates that the phenomenon is quite complicated. For example, Coulson has noted three additional factors that contribute to the dipole. These derive their interpretation from the molecular orbital considerations. If the bonded atoms are different in size, there is a resulting contribution to the dipole. If the atomic orbitals involved (through overlapping) in the formation of the bond are hybridized, this contributes to the total dipole moment of the bond—and practically all bonds involve a degree of hybridization. Finally, if there are unshared electrons present on one of the bonded atoms, there will be an interaction between these and the bond electrons which will lead to a polarization of the bond—an effect which may be very great in some

cases. These factors usually combine in such a way, in influencing the molecular orbitals of the bond, that the resulting bond dipole moment is at a minimum. Where considerations such as these latter ones are important (as they may be in understanding induction effects) they will be mentioned. Otherwise, we will take the least complicated view possible.

The **coordinate bond,** since it results from the sharing of a pair of electrons both of which were in effect donated by one of the bonded atoms (no matter how the bond was actually formed), is the seat of a *strong permanent dipole:*

$$R-\underset{\underset{\displaystyle O}{\downarrow}}{\overset{\overset{\displaystyle O}{\uparrow}}{S}}-OH; \quad \overset{\delta+}{R_3N} \rightarrow \overset{\delta-}{O}$$

Although the bond between the atoms may be a strong one, *directed between the bonded atoms, it also exhibits a strong, permanent "undirected" electrostatic bonding power.* A coordinate bond is usually indicated by an arrow, showing the direction of sharing of the electron pair, as in the symbols above.

In summary, then, the dipole moment of a bond is the manifestation of a partial ionic character ($\delta+$, $\delta-$) in the bond. In any bond between a pair of atoms (A,B) for which we can write the symbols $\overset{\delta+}{A}-\overset{\delta-}{B}$, or $\overset{+\!\!\longrightarrow}{A-B}$, or $A \rightarrow B$, the bonding ability, the affinity, will be less localized between the two atoms than in a pure covalent bond. Such a bond will exhibit a degree of electrical dissymmetry. It is this electrical dissymmetry, this "unused bonding power," at a bond that gives the molecule some of its chemical and physical reactivity. Molecules that contain such bonds may be called *polar molecules*, whether or not they exhibit a molecular dipole moment (see below).

III. PHYSICAL INTERACTIONS

The "physical" interactions between molecules that are reflected in bulk phenomena of interest to chromatographers can be understood in terms of the approach already taken. These interactions result from the presence in molecules of bonds of the kinds described above. A molecule that contains bonds of several different types would show several types of physical interaction. The physical interactions that are of particular interest are those leading to attractive interaction: the dispersion, the orientation, the induction, the ion–dipole, and the hydrogen bond interactions.

The **dispersion interaction** may be described in terms of simple valence theory. Since the electrons in atoms are always in motion, there will be times when, if they could be instantaneously examined, they would be found unsymmetrically placed, and hence one would recognize a sort of instantaneous dipole. However, at the next instant this would have developed into some other dipole, and when looked at over a short time interval, these successive "instantaneous" dipoles would be found to have canceled out into a nonpolar composite. Homonuclear molecules such as A—A would be nonpolar; heteronuclear molecules such as A—B would be to some extent polar. Now two molecules approaching each other might find their "instantaneous" dipoles moving in phase, so that though the molecules themselves may be nonpolar, or essentially nonpolar, yet there would arise an attractive interaction between them owing to the movement in phase of their electronic systems: a mutual interaction of the fluctuating dipoles. This effect can be additive over large numbers of molecules in a system and is thought to be the force largely responsible for adhesions between such otherwise inert structures as hydrocarbon molecules (for example). It is likely to be most effective between like molecules. The subject has been discussed by Lennard-Jones (537) and London (563).

The dispersion interaction would be likely to be most important in attracting like types of essentially nonpolar molecules to each other. As an example, we would expect that when volatile paraffin hydrocarbons are separated from each other by gas partition chromatography (Chapter V) using mineral oil as the stationary phase, the observed *retardation* of higher molecular weight components is due to the stronger dispersion interaction of the longer chains with the paraffin molecules of the stationary phase. The volatility of the longer chain molecules would be less than that of the shorter chain from this stationary phase.

The **orientation or dipole–dipole interaction** occurs between molecules with permanent dipoles. If the molecules come into contact in a suitably oriented manner, with the like poles oppositely placed, thus:

$$\begin{array}{c} \overset{+\longrightarrow}{\mathrm{A} - \mathrm{B}} \\ \overset{\longleftarrow +}{\mathrm{B} - \mathrm{A}} \end{array}$$

an attraction results between them. At ordinary temperatures such an orientation would be preferred, but the interaction is a weak one and is readily destroyed under the buffeting that occurs at higher temperatures. Associations leading to departure of bulk phases from ideal behavior have been laid at least partially to this type of interaction. For example, negative deviations of mixtures from Raoult's law imply attractions between solvent and solute molecules, and this corresponds with an ob-

served increased solubility (increased over the ideal) of the solute in such mixtures. Where the components of such mixtures are polar, then part of the observed bulk behavior would be expected to be due to orientation interactions.

The **induction interaction** may occur when a molecule with a permanently dipolar bond, and a nonpolar molecule (or group in a molecule) with a relatively mobile electron system approach each other. The permanent dipole induces a temporary dipole in the other system so oriented that attraction interaction can occur. For example:

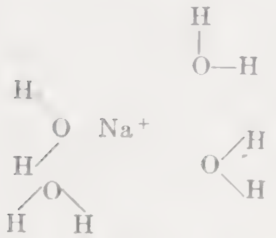
$$\overset{+\longrightarrow}{\mathrm{A}-\mathrm{B}} + \mathrm{C}::\mathrm{D} \longrightarrow \begin{array}{c} \overset{\delta-\;\;\delta+}{\mathrm{C}::\mathrm{D}} \\ \overset{+\longrightarrow}{\mathrm{A}-\mathrm{B}} \end{array}$$

An ion may interact inductively with such a molecule, and often induction interaction leads to chemical reaction.

$$\mathrm{H}^{+} + \;\rangle\mathrm{C}{=}\ddot{\mathrm{O}}{:} \longrightarrow \;\rangle\mathrm{C}{=}\overset{+}{\mathrm{O}}\mathrm{H} \longrightarrow \;\rangle\overset{+}{\mathrm{C}}{-}\ddot{\mathrm{O}}{:}\mathrm{H}$$

There are many chromatographic examples of the influence of induction interactions on separation. (Note that induction interaction must not be confused with the inductive effect of a group on the rest of a molecule.) For instance, the separation of paraffins, olefins, and aromatics on silica gel could be explained on this basis. What is observed is that on a column of silica gel, the paraffins are least strongly held, the olefins next, and the aromatics most, with 3-ring more strongly held than 2-ring, and these than 1-ring, aromatics. The interpretation is that the electrons of the aromatics, and less so of the olefins, respond to the permanent dipoles of the silica gel, $\overset{\delta+}{\mathrm{Si}}{-}\overset{\delta-}{\mathrm{O}}{-}$, by being displaced to produce temporary dipoles oriented as shown above, to yield attractive interaction. The larger the number of condensed aromatic rings the more extensive the interacting system. (Of course, geometrical considerations play a role, in that some molecules may be too large to enter pores in the gel that are readily accessible to others, but this is not the question here. Cyclohexane is in any case less strongly retarded by the gel than benzene.)

The **ion–dipole interaction** occurs between an ion and a permanent dipole in a molecule, so that an attraction (or repulsion) occurs. Familiar examples are solvations of ions. Water, ammonia, alcohol, and other molecules contain permanent dipoles, and these molecules will "solvate" ions. For example,

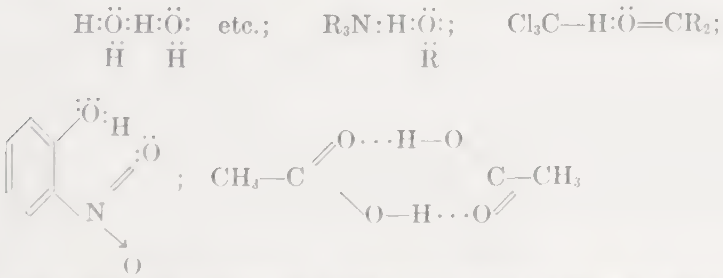


Sometimes this interaction is so strong that the products are stoichiometrically well characterized, in which case the binding may be called chemical. For example, the first molecule of water that interacts with a proton, or the 4 molecules of ammonia with a divalent nickel ion, may be designated as chemically combined:

$$H^+ + H_2O \longrightarrow H_3O^+ \text{ or } Ni^{++} + 4NH_3 \rightleftharpoons Ni(NH_3)_4^{++}$$

In other cases, the interaction occurs with a lower release of energy and it is designated as physical. An example of this would be the hydration of sodium ion, illustrated above.

From the chromatographic point of view, probably (and certainly from the *paper* chromatographic point of view) the most important polar interaction is the **hydrogen bond.** This may occur between molecules or within molecules. A hydrogen bond may occur between a molecule with hydrogen attached to F, or O, or N, or in certain cases C, and a molecule with unshared electrons. The hydrogen mediates the bond. Examples are:



In general, the hydrogen bonding tends to *depolarize the molecule*, using up, so to speak, its interacting power. For example acetic acid in the dimeric and trimeric forms is soluble in such nonpolar solvents as benzene and other hydrocarbons. This is the evidence that defines this expression "depolarization." In effect, the hydrogen bridging power of the carboxylic acid group is largely used up in the dimer and trimer so that the resulting complex is less polar than the monomeric molecule. The observed solubility in a nonpolar hydrocarbon gives evidence for the nonpolar state of the complex. Similarly, internal chelation, as shown in o-nitrophenol (above), results in depolarization. This is shown by the

fact that the ortho isomer is lower melting and more steam-volatile than the para isomer, which cannot chelate internally. These observed properties also serve to define the depolarization that has occurred, since they give evidence of lowered intermolecular interaction in the ortho compound.

Evidence for the role of hydrogen bonding in all kinds of chromatography is widespread and will be frequently adduced.

IV. MEANING OF TERM "POLAR"

At this point we must state explicitly what we mean when we use the term "polar." This word has been given many different meanings, one of which has been correlated with "having a dipole moment." It has been rejected in this use as a tool for correlating solubility with dipole moment, as being too crude and inadequate (401).

We wish to use the term polar to refer to a bond, or an ion, or a molecule, or a phase, always qualifying the use if there may be any doubt about it. By a **polar bond** we mean a bond that exhibits an electrical dissymmetry such that interactions of orientation, or induction, or ion–dipole, or hydrogen-bridging types, or combinations of these may be entered into. The "bond" includes the bonded atoms, since the overlapping orbits that are the bond circumscribe both bonded nuclei. An ion is by definition polar, so it would be redundant to say "polar ion." A **polar molecule** is a molecule that exhibits the above interactions by virtue either of an overall molecular dissymmetry (in which case it will show a dipole moment) or by virtue of a localized polar bond (in which case the molecule may show no dipole moment). A **polar phase** is one composed of polar molecules, and is distinguished from a nonpolar phase by the fact that the two will be "not" miscible or only partly miscible.

All these terms have operational definitions and are commonly accepted though sometimes rather loosely used. The operational definition of a polar phase was given in the previous paragraph. Other names for polar and nonpolar substances (molecules, or phases) are "aqueous" and "organic"; or hydrophilic and lipophilic. These names derive from observations made during the working up of the substances—as by extraction with ether from a water solution or suspension. The "organic" or lipophilic substances go into the ether. We will usually avoid these terms, and use *polar* since this carries the connotations to the molecular and atomic level.

A polar molecule—a molecule that shows polar behavior—may or may not exhibit a dipole moment, and conversely, some molecules with polar bonds may act in a quite nonpolar manner. For example, a substance like paradichlorobenzene has no dipole moment. Yet, if it were adsorbed on a surface, lying flat, the interactions with the molecules in that surface

might well be quite localized at the bonds. These bonds, then, behave in a very polar (in our present sense) manner. Large molecules will usually interact with a surface on only one "side," so that it is the polarity of available bonds that must be considered, as well as the geometry of the molecule, in predicting adsorption. Similarly, as Hildebrand and Kufaru pointed out, moderate dipoles, especially those that may be somewhat buried in a molecule, may have little effect on the overall polarity of the molecule. Thus chloroform, and ether, though containing polar bonds, are yet good solvents for nonpolar molecules and thus behave in a nonpolar manner.

Hence our designation "polar" is given to a bond, molecule, or phase on the basis of chemical knowledge from considerations of *chemical behavior* or *molecular structure*. This is justified by the fact that the chromatographer rarely knows the dipole moments or dielectric constants, or such parameters of the substances in the mixtures he wishes to separate, but he almost always knows something about the chemical nature of the mixture. This information was derived during the working up of the mixture and sometimes can suggest the types of groups present, such as water-soluble or fat-soluble. (See Chapters XIV and XV.)

It should be further stated that in using the term "polar" we shall normally have a relative relation in mind. We may, for example, state that the carboxylic *group* at the end of a normal fatty acid, or the hydroxyl group of an alcohol, is *more polar than* the rest of the molecule, and hence that this is a more interactive site in the sense defined. Or we may say that a lower fatty acid *molecule* is more polar than a higher fatty acid molecule because in the latter the influence of the nonpolar paraffin chain is more evident than it is with a lower fatty acid. An experimental basis for such a distinction is that when fatty acids are distributed between a polar and a nonpolar phase such as water and benzene, the lower acids are found to go more into the water than into the benzene, and the higher acids more into the benzene than into the water.

Finally, we may refer to one *phase* as being more polar than another, e.g., water than amyl alcohol; silica gel than benzene; and so on, meaning that it is more capable of associative or other interactions than the other phase.

V. CONCLUSION

This brief summary of chemical and physical interactions is given as a convenience, since we will frequently refer to these interactions in the course of the following chapter, and we can in this way be most explicit. Not only are R and R_F values more understandable when the structures

and interactions of the molecules that are present are taken into consideration, as was referred to in the case of gas chromatography, but also the distortions of zones can in many cases be understood. For example, excessive streaking, or tailing in paper chromatograms, and tailing of zones in columns; double-zone formation from a single substance; spots or deposits at the site of the initial mixed zone; R or R_F values of zero, or 1.0, can be understood, and in many cases remedied, through a consideration of interactions at the molecular level. There are, of course, many phenomena in chromatography that are not at all understood. There are others whose explanation must clearly be sought at the bulk level of phases and interfacial phases; of viscosity, density, and similar properties of bulk matter.

But it must be expected that ultimately all these phenomena will become understandable in terms of the atoms and the ways they are combined into molecules, and of the molecular interactions that reflect the composition and constitution of the mobile phase, stationary phase, and zone substances of the chromatographic system.

General Theory

Theory has been developing quite steadily in all areas of chromatography. An examination of the state of this theory leads to the following conclusions:

1. In all areas, theory is of practical value for the purpose of organizing data and the relationships of data.

2. In some areas, detailed theory is of value in indicating mechanisms of the processes that are occurring.

3. In some areas, detailed theory is not of practical value to the person faced with a new separation problem: that is, with a problem in which he has to work with mixtures of scarcely known composition or with mixtures of newly met substances.

4. All separation problems that depend on the distribution of substances between phases may be approached with the same type of theoretical treatment, but each may be best suited by a particular treatment.

I. DEFINITION OF GENERAL THEORY

By theory we mean primarily the quantitative description of chromatography in terms of the detailed phenomena occurring during the separation process. However, the term may be extended, where such quantitative description is impractical, to a more qualitative description. Here the theory would rest on an analysis of the methods, based in the first instance on the quantitative, detailed theory, but using it only to derive an organizational plan for studying the less well-understood particular practical problems. It is this latter approach that we term *general theory*. We imply a treatment that draws together the different kinds of chromatography and explains them in terms that are useful to the working chromatographer: that enables him to understand his observations and make the predictions that can lead to improved separations.

We can generalize the distribution relationships for all chromatographic systems (adsorptive, ion exchange, partition, and foam or emulsion) for the purposes of this chapter. In all cases, *one phase is mobile*, and moves in a countercurrent manner over the other, which is usually fixed or essentially nonmobile: *the "stationary" phase*. In effect, of course, as well as in practice in some chromatographic applications, the two phases move

countercurrently to each other. But in most cases, a bulk mobile phase, **M** (the *solvent*, *developer*, *eluent*, or *displacer*, depending on how it functions) moves over a stationary phase, **S**, which may be an *adsorbed surface phase*, or the *surface* of *an ion exchanger*, or a *sorbed "liquid" film*, or a *"mobile" surface phase* at the boundary of a bubble or droplet. In this last case "mobile" means that the phase is one between two fluids, and not that it undergoes gross flow. When we use the term mobile, unqualified with quotation marks, we will always mean a phase that undergoes or may undergo bulk flow. The terms mobile and stationary phases will be utilized in this chapter because of their generality. They may be particularized for each kind of chromatography as shown in Table II-1.

In general, the mobile phase is a bulk phase, whereas the stationary phase is essentially two-dimensional, or at least of much greater extent than thickness. For this reason geometric factors are more important in the behavior of the stationary than of the mobile phase.

The distribution of solute thus occurs during the chromatographic process between mobile and stationary regions:

Solutes in solution	:	Solutes in stationary phase
(components of phase **M**)		(components of phase **S**)

This is the key phenomenon that ties all kinds of chromatography together. The practical application of chromatography depends on influencing this distribution process, so as to drive the solutes selectively into one or the other phase at the proper stage in the separation process.

II. PRACTICAL CONSIDERATIONS

Practically speaking, the chromatographer is interested only in readily measurable and manipulable factors that affect the separation. Thus although it is possible to write equations to describe a simple chromatographic separation in detail and to solve them, the data necessary to accomplish such a tour de force are usually lacking to the practical chromatographer.

The most important readily measurable parameter that is directly connected with the separation process is the R or R_F value of the zone: the velocity of movement of the zone along the column or sheet divided by the velocity of movement of the mobile phase. Returning to the relationship

Substances in **M** : Substances in **S**

it is evident that if a substance, 2, tends to distribute itself more to the mobile phase than another substance, 1, does, it (2) will move more rapidly and show a higher R or R_F value than will 1. The substance, 1, which

tends more to the stationary phase, will be more retarded, and show a lower R or R_F value than will 2. This was illustrated in Fig. II-1.

The distribution of a substance between two phases is governed by the molecular interactions within the system (Chapter III). It is reflected by an equilibrium coefficient α, which is a function of the equilibrium concentration of the substance in the stationary phase, $C_S^{\text{equil.}}$, divided by that in the mobile phase, $C_M^{\text{equil.}}$:

$$\alpha = f(C_S^{\text{equil.}}/C_M^{\text{equil.}}) \tag{1}$$

The concentrations $C_S^{\text{equil.}} + C_M^{\text{equil.}}$ represent the total amounts of the substance regardless of molecular state in the respective phases, per unit volume. These are analytically determined amounts of substance, and

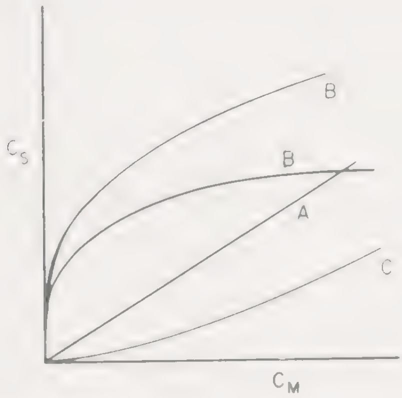


Fig. IV-1. Types of distribution isotherms in the lower ranges of concentration. The substance is distributed at equilibrium and constant temperature. The stationary phase, or in a batchwise liquid–liquid distribution, one of the phases S, contains the substance in stoichiometric concentration C_S. The other, or mobile phase M, contains the substance in the concentration C_M. The distribution coefficient α, where $C_S = \alpha C_M$, is a constant in the case of curve *A*. Curves of the *B type* may take forms fitted by the Freundlich isotherm, $C_S = KC_M^n$, where K and n are constants; of the Langmuir type, $C_S = k_1k_2C_M/(1 + k_1C_M)$, where k_1 and k_2 are constants; or other forms. Curve *C* is the inverse of curve *B* in its general *type*.

include any forms produced by association or dissociation equilibria. In the case of ion exchangers the expression is usually not so simple.

III. DISTRIBUTION CURVES

C_S will have a fixed value at any given equilibrium concentration, C_M, and so will α (687). But α may change with change in concentration. Three general *types* of behavior of α have been observed (Fig. IV-1). The ratio α may be essentially constant over a range of concentrations, as shown in

curve A. Curve B illustrates the situation where the concentration in the stationary phase is *relatively* higher the lower the concentration in the mobile phase. This type of isotherm is fairly typical of adsorption distributions. It is also found in liquid-liquid and vapor-liquid distributions. Curve C is the inverse of Curve B. In this figure the actual shapes are not meant to be more than illustrative of the *type* of behavior observed.

When curves of type B occur in liquid–liquid or gas–liquid distribution (in the latter the stationary phase is a liquid sorbed to some support such as diatomaceous earth or paper), the shape of the curve is ascribed to the existence, in one phase, of an equilibrium between the solute molecules whose concentration is being measured and other species which are the result of association or dissociation of the solute. These other species may or may not distribute, and the amounts of them formed may not bear a constant relation to the concentration. For example, if the stationary phase were aqueous, and the mobile phase organic, and the solute a weak organic acid, a curve of type B might be observed. At low concentrations the acid will be more extensively ionized and thus more soluble in the stationary aqueous phase. Organic liquids such as ether or benzene are not good solvents for ionic species. There might be some of the organic acid in the wet organic phase, where it might hydrogen bond to the organic molecules or interact via the water molecules present. The acid may also be dimerized or trimerized in the organic phase through hydrogen bonding. But the acid will tend more to the aqueous phase at lower concentrations if it is water-soluble.

At higher concentrations, the per cent ionization will be less in the aqueous phase, and relatively more unionized molecules will be available for distribution equilibrium with the organic phase. Thus at higher concentrations the curve will tend toward the C_M axis.

In the case of adsorptions, curves of type B may have their origin in the same types of phenomena especially when adsorption to the surface of a solution of an ionized adsorptive is the phenomenon in question. In addition, where the adsorption is to a solid adsorbent the relatively high extent of adsorption at lower concentrations may be ascribed to the presence of highly "active" sites in the inhomogeneous surface of the adsorbent, which are preferentially covered even at low concentration. As the surface is covered at higher concentrations relatively less and less adsorptive can be taken up and the curve tends toward the C_M axis, becoming parallel with it if the surface of the adsorbent becomes saturated.

Curves of the type C (Type III of Brunauer *et al* (129), see Fig. IV-12) occur in adsorption systems when the adsorption is weak, but the attraction between the adsorbent and the adsorbed molecules, even though

small), is sufficiently great to supplement a tendency of the molecules to aggregate (condense, or crystallize) on the surface, so that with increase in concentration they form clusters and eventually thicker layers on the surface of the adsorbent. The interpretation of gas adsorption isotherms has been discussed by Brunauer(128).

It will be noted that at low enough concentrations all these curves approach linearity.

IV. BATCH AND CASCADE PROCESSES

1. Liquid–Liquid Distribution

If a certain weight of substance in a certain volume of liquid **M** (concentration C_M) is shaken in a separatory funnel with an equal volume of another liquid S, both liquids being "immiscible" and mutually saturated before these manipulations, a portion of the substance will pass reversibly from solution in **M** into solution in S. At equilibrium, the relation $\alpha = C_S / C_M$ will hold. (We will neglect possible influences of the solute on the solubility of liquids **M** in S, and S in **M**; and possible volume changes.) This is a single-step distribution or a *single-batch* process (Fig. IV-2).

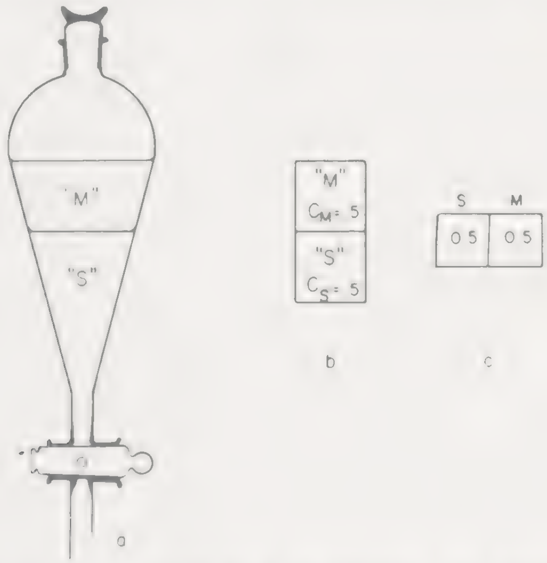


Fig. IV-2. Batch process. Single-stage distribution. (*a*) Example of a liquid–liquid extraction, where **M** may be ether (say) saturated with water, and S water saturated with ether. (*b*) Generalized statement of the concentration relations in only two phases in contact at equilibrium, when $\alpha = 1$. (*c*) The diagram *b*, which might have represented the separatory funnel in *a*, is turned through 90° for later convenience (Fig. IV-3). The square represents a unit volume of the phase, and the figure the concentration of solute or adsorptive. Again, in *c*, $\alpha = 1$.

A *cascade* type of distribution involving four steps is represented in Fig. IV-3. Imagine a row of four separatory funnels containing one unit volume each of stationary phase. These are funnels 1 to 4. Imagine also a set of flasks containing one unit volume each of mobile phase; these are *A* to *D* (Fig. IV-3,*a*). The mobile and stationary phases have been

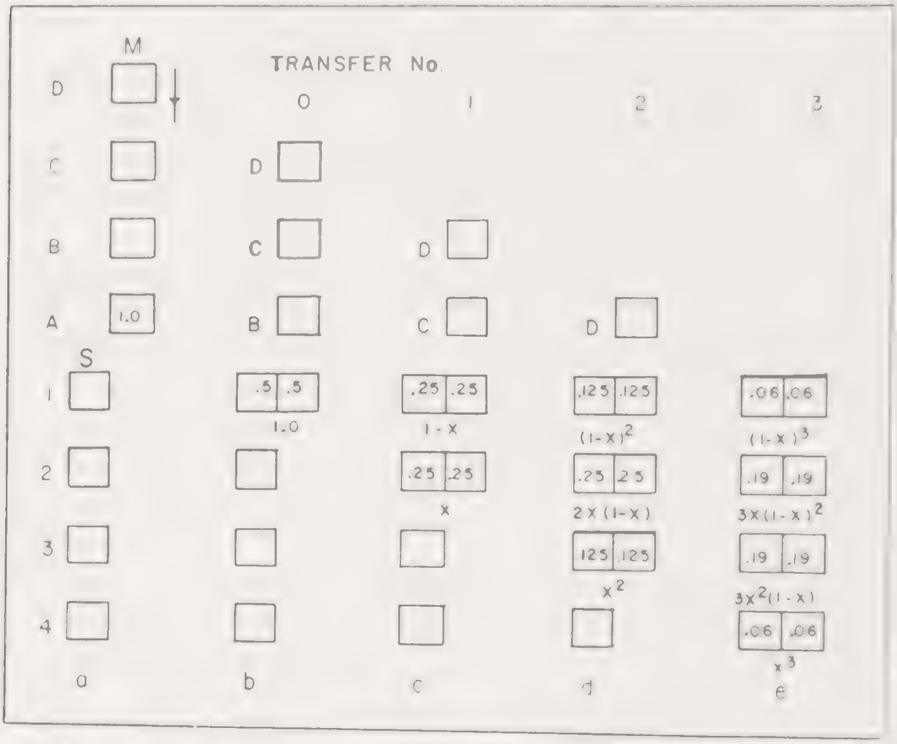


Fig. IV-3. Cascade process. A cascade type of equilibrium distribution of a substance between two phases **S** and **M**. A square represents a unit volume of a phase (saturated initially with the other phase), and it is assumed that the volumes are not appreciably affected by the presence of the solute. Presence of the solute is indicated by numbers in the boxes. The rectangles may represent separatory funnels (as in Fig. IV-2) or flasks containing solution and adsorbent, or apparatus containing other pairs of phases. The figures are given for a solute with a distribution coefficient $\alpha = 1$, constant with change in total concentration. The scheme is generalized, where x is the fraction going to the mobile phase, and the *total* concentration of solute in each stage is given below the boxes. See text. (Adapted from Craig and Craig (108), Fig. 7.)

equilibrated by shaking them together until there is no further change, and then they have been separated and measured respectively into the funnels and flasks. Thus they are mutually saturated. One unit of solute is dissolved in flask *A* and it is assumed that there is no volume change. For purposes of concrete illustration, it is taken that the distribution coefficient of this substance is 1, and does not change with changes in

concentration. In the illustration the amounts of material in the flasks and separatory funnels are also generalized to other values of a by the device of fractions, $a = (1 - x)/x$, and it is assumed that a is constant.

The phase **M** in flask A, containing solute, is transferred to the funnel 1, containing phase **S**, and shaken until equilibrium is reached. The situation is represented by step b. In each layer there will be 0.5 of the original amount of the substance, since $a = 1 = 0.5/0.5$. Generalized, the **S** phase contains $(1 - x)$ and the **M** phase (x) of the original amount of substance, the *total* in this funnel being 1.0. This might be likened to an initial mixed zone of a substance that distributes with this a between mobile and stationary phases of equal volume per unit thickness of the zone.

Now the **M** layer in this funnel (No. 1) is transferred to funnel 2, containing empty **S** phase, and then fresh, empty **M** phase is transferred from B to replace the **M** phase removed from funnel 1. The two funnels 1 and 2 are now shaken to equilibrium, and the result is step c. Each funnel now contains a total of 0.5 of the substance, equally distributed ($a = 1$) between each layer. This will be called the first transfer.

In the next step the procedure is the same: **M** layer in funnel 2 is transferred to funnel 3, where it becomes distributed equally between both layers; **M** layer from funnel 1 is transferred to funnel 2, which already contains solute, and the total is distributed equally between both layers; and fresh, empty **M** from flask c is transferred to funnel 1, whereupon the solute that was in the **S** layer distributes between the two. The result is shown in step d, the second transfer. The procedure, repeated once more, yields step e, the third transfer.

The results of this procedure may be plotted as shown in Fig. IV-4, where the total weight of solute in each funnel is plotted as a fraction of the original weight of substance, against the number of the funnel.

The implications of such distributions and the various ways the procedures may be run are documented and given in great detail by Craig and Craig (198). The remainder of this section is largely taken from their discussion.

It can be seen from Fig. IV-4 that during this procedure (*1*) the substance is spread out, along the row of funnels, in the direction of motion of the mobile phase; and (*2*) the peak of concentration is moved in the same direction. It is not necessary to spell out the further steps in such a process, for they can be obtained from an expansion of the relation: (fraction in stationary phase + fraction in mobile phase)^n = 1, where the "fraction" is the fraction of the original amount applied, and n is the number of the transfer, beginning with the first, e.g., step c in Fig. IV-3. Each funnel corresponds to a term in the binomial expansion above, when it contains both phases. Thus there are 4 terms at the third step.

Fig. IV-4. Plot of distribution shown in Fig. IV-3, for liquid–liquid distribution. The *total* fraction of substance in a funnel is plotted against the number of the funnel. The fraction comprises the total weight of solute in both layers divided by the original weight of solute that was started with. The letters *b* to *e* refer to the steps in Fig. IV-3. *F* is fraction in funnel; *N* is number of funnel.

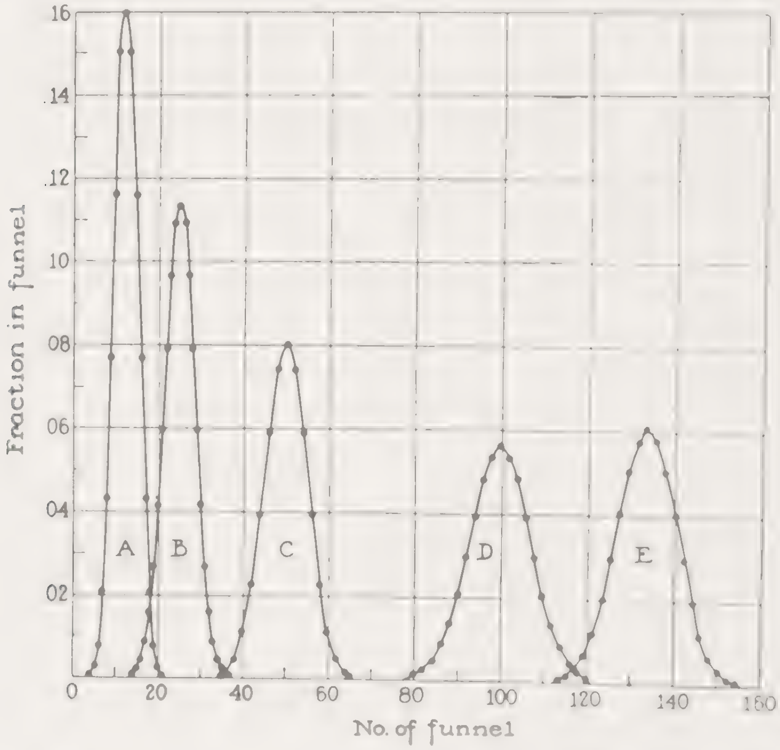


Fig. IV-5. Distribution patterns for large numbers of stages. The area under the curve shows the amount of substance, the position of the curve shows the location of the zone of substance, and the height of the curve at any given point shows the concentration in the zone at the point. (From Craig and Craig (108).)

When such an expansion is carried out for larger numbers of stages, the broadening of the zone of substance in the funnels and the movement of the region of peak concentration becomes quite evident, as the Craigs have

shown (Fig. IV-5). The results for distribution ratios greater and less than 1.0 are compared with $\alpha = 1$ in Fig. IV-6. It can be seen that with $\alpha = 3.0$ (curve 2) the peak is retarded, compared with $\alpha = 1$ (curve 1). This is understandable in that a high α, where the relation is $\alpha = C_S/C_M$, describes a substance that tends more to the stationary phase. Conversely, with a lower α of 0.333 (curve 3), the peak moves farther than for $\alpha = 1$.

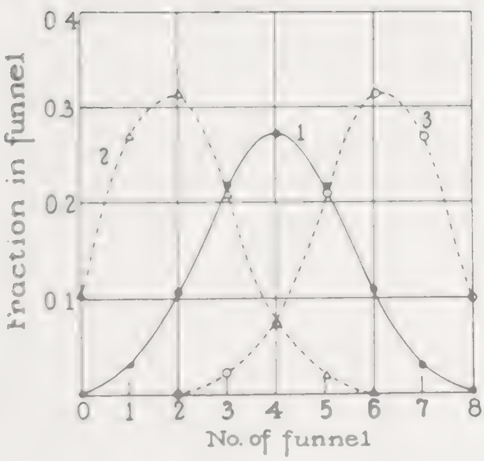


Fig. IV-6. Distribution curves for different values of α. Each curve shows the pattern for a substance with that distribution constant after eight transfers. The constants are, for curve 1, 1.0; for curve 2, 3.0; for curve 3, 0.333. (From Craig and Craig (198).)

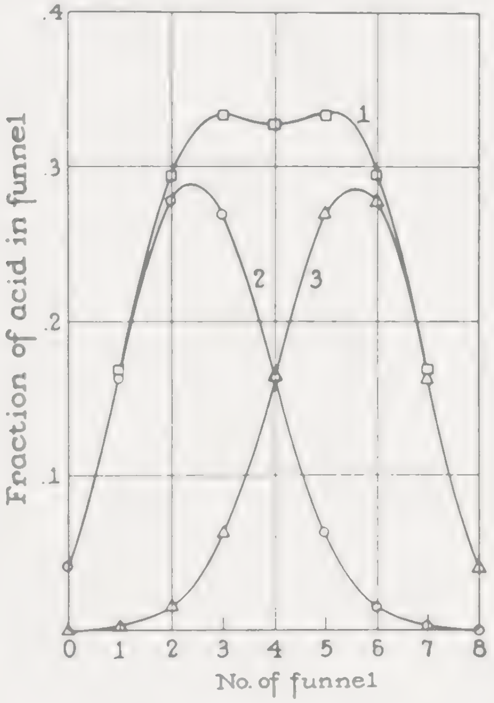


Fig. IV-7. Distribution curves and pattern for a mixture of propionic and butyric acids. The pattern is shown after transfers. The two acids were distributed between isopropyl ether (phase **M**) and an aqueous buffer (phase **S**) the pH of which was adjusted so that $\alpha_{propionic} = 1/\alpha_{butyric}$. $\alpha_{propionic} = 0.485$; $\alpha_{butyric} = 2.06$. The zones move in an essentially independent manner. Curve 1 shows the values of total acids in the various funnels. (From Craig and Craig (198).)

Carrying this illustration one step farther, by using a mixture of two solutes, the curves of Fig. IV-7 are obtained. This represents the partial separation of propionic and butyric acids in an eight-stage distribution, using an aqueous buffer (phase **S**) and isopropyl ether (phase **M**, in our

convention). The authors used a buffer adjusted so that the geometric mean of the a's of the two acids in this system is 1, since this condition gives the best separation. In calculating the curves more complicated equations involving the a's of the two acids must be used. These are discussed by L. C. Craig and D. Craig (198). For a general treatment see also Klinkenberg and Sjenitzer (481a).

2. Adsorption

The discussion so far has been designed to give a simple picture of a *single-batch* process and its extension to a countercurrent *cascade* process. The example taken was that of liquid–liquid distribution. The same type of approach may be used with adsorption. However, here a curve of the type B is the rule, and a is not constant with change in concentration. (At very low concentrations a may be so nearly a constant that it may justifiably be treated so and the above approach may be used.)

Many empirical, as well as theoretically founded, equations have been set up to deal with curves of type B (7,299,899). An equation that fits a great many adsorptions from solution has come to be known as the Freundlich equation (299):

$$\mathbf{C_S} = K(\mathbf{C_M})^n \tag{2}$$

where K and n are constants, and n is less than 1. In this equation, $\mathbf{C_S}$ is usually determined as a "specific amount adsorbed," that is, x/m, where x is the weight, or number of moles, etc., of adsorptive adsorbed by m grams of adsorbent. In distinction to the Langmuir equation (equations (6a,b) and (8)), this equation does not reduce to a linear form at very low concentration, nor does it approach a constant value for the adsorption at higher concentrations. Data fit this equation over the range where a plot of log (x/m) against log $\mathbf{C_M}$ yields a straight line:

$$\log (x/m) = \log K + n(\log \mathbf{C_M}) \tag{3}$$

The intercept is log K and the slope is n. The Freundlich equation will be used to illustrate what follows (560). Other equations will be discussed below.

In ordinary, batchwise adsorption operations, a quantity of adsorbent, m, is mixed with a quantity of solution, v, of concentration c_0. The process of adsorption which takes place serves to deplete the solution, so that its concentration falls to a new value, c. The amount of substance adsorbed is, to a close enough approximation, the amount which has disappeared from solution, $(c_0 - c)v$. (The reason that an approximation is involved is that solvent is also adsorbed.) The process may be allowed to reach

equilibrium, in which case the amount of substance adsorbed, x, has a maximum value and may be related (at a given temperature) to the equilibrium concentration $c^{\text{equil.}}$ by means of the adsorption isotherm $x = mf(c^{\text{equil.}})$. The important point here is that the adsorbed substance comes into equilibrium with a solution which has a concentration less than that of the original solution, since the act of adsorption depleted the solution. It is apparent, from an examination of an isotherm, that for a given weight of adsorbent relatively fewer molecules will be adsorbed at the concentration c than could be adsorbed if the equilibrium concentration were higher, say c_0, where x_0/m would be adsorbed (Fig. IV-8). The

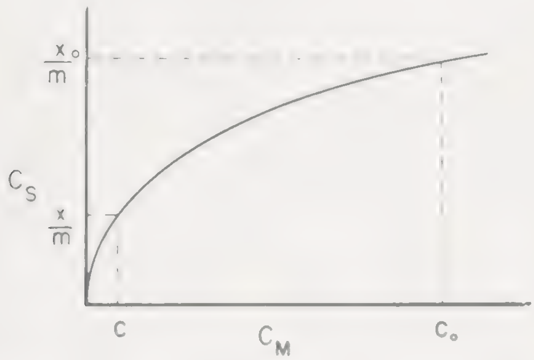


Fig. IV-8. Freundlich adsorption isotherm. Adsorption isotherm for a substance that obeys the Freundlich relation $v(c_0 - c)/m = Kc^n$. Here v represents volume of solution of concentration c_0, which is treated with a mass m of adsorbent. The equilibrium concentration is c; K and n are constants. The specific amount of substance adsorbed is x/m; then $x/m = Kc^n$.

batch operation is completed by separating the solid and liquid phases. The solid carries with it the adsorbed molecules and some solvent. The adsorbed molecules can be recovered by elution.

Sanders (1904) improved the efficiency of the simple batchwise adsorption operation by an *explicit* application to it of the principle of "countercurrent action." Sanders was interested in decolorizing sugar solutions. In this operation it was sufficient to reduce the "color" from an original value c_0 to a definite, low value c. The adsorption isotherm for the colored material (the "color") was described quite well by the Freundlich isotherm, which could be written in the form:

$$v(c_0 - c)/m = Kc^n$$

Here $v(c_0 - c)$ is the amount of color removed by m grams of decolorizing charcoal, and K and n are constants. It is possible to calculate, on the basis of the isotherm, how much charcoal need be applied to a given

volume of solution to reduce the color from c_0 to c in one-step, two-step, or three-step, etc., operations. The operation considered is such that fresh adsorbent enters the system and comes into contact first with partly decolorized liquid, reducing its concentration to the final low value c. The partly spent charcoal is then used stepwise against progressively less decolorized liquids until it emerges from the system as completely spent as possible. The operation for a three-stage system is illustrated in Fig. IV-9, and the process is analogous to that illustrated in Fig. IV-3. Only the apparatus is different. The calculations for Sanders' method are not given. The results of the calculations for a particular isotherm are gathered into Table IV-1. It should be mentioned that in starting such a

TABLE IV-1
Comparison of Single-Stage and Multiple-Stage Use of an Adsorbent
The isotherm is $v(c_0 - c)/m = 1.2c^{0.32}$ [or $C_S = 1.2(C_M)^{0.32}$]

Operation	Amount of adsorbent used (g.)
One-stage	1.0 m
Two-stage	0.436 m
Three-stage	0.366 m
Four-stage	0.347 m

system as this the initial batch of charcoal is not used in a countercurrent way. The calculations are for a system which is already in operation.

When the three-stage system is in operation (Fig. IV-9), fresh adsorbent of quantity 0.366 m (in the case illustrated) enters the system first into contact with liquid of concentration c_2 (i.e., liquid which is already partially decolorized). This fresh adsorbent is in sufficient quantity to lower the concentration of the solution to an equilibrium value c, at which point the amount of color adsorbed on the charcoal is $(c_2 - c)v$. The adsorbent is separated from the solution and brought next into contact with liquid of concentration c_1, from which it adsorbs a quantity $(c_1 - c_2)v$ of color and brings this solution to an equilibrium concentration of c_2, itself having a total quantity x_2 of solute adsorbed upon it. The mixture is then separated, and the solution of concentration c_2 passes on to meet a fresh batch of adsorbent. The partly used adsorbent is next brought into admixture with the fresh solution of concentration c_0 and is able to bring its concentration to c_1 by adsorbing $(c_0 - c_1)v$ of color. The adsorbent then leaves the system in equilibrium with solution of concentration c_1 and carrying adsorbed upon it x_1 of solute.

When the three-stage is compared with the single-stage operation it becomes evident why less adsorbent is needed in it, for in the case of the

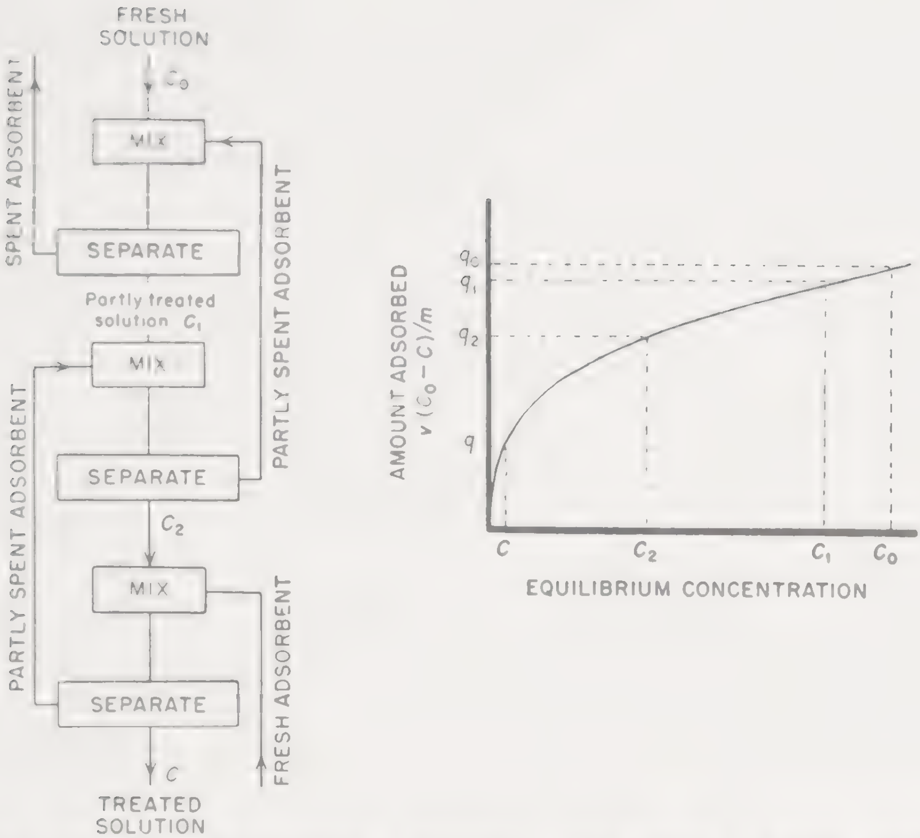


Fig. IV-9. Cascade application of adsorption. The isotherm is shown, together with a flow diagram of the operations for a three-stage process. The spent adsorbent leaves the system with a concentration c_1, after having adsorbed an amount q_1 of adsorptive per gram. The treated solution leaves at concentration c, having been brought to this value by fresh adsorbent.

three-stage operation the adsorbent leaves the system in equilibrium with a solution of the relatively high concentration c_1, whereas in the single-stage operation it leaves at a concentration of c. Thus, mass for mass, the adsorbent removes more solute in the former than in the latter case, and hence to remove a given amount of solute less adsorbent is used in the former than in the latter. The calculation involves figuring the amount of adsorbent needed so that:

$$(c_0 - c_1)v = x_1 - x_2; \quad (c_1 - c_2)v = x_2 - x; \quad (c_2 - c)v = x;$$
$$(c_0 - c)v = x$$

After the system is in operation, the greater the number of stages in which the adsorbent can be applied the less is the amount of charcoal needed to produce a given decolorization. Sanders showed also that the

greater the percentage of substance which is to be removed, that is, the further toward zero is to be the final concentration c, the more efficient is the countercurrent compared with the single-stage operation.

V. THE CHROMATOGRAPHIC PROCESS

In chromatography the process is a differential countercurrent one, but the same *type* of reasoning as followed above is applicable. The difference is that, as the Craigs put it (198), the mathematics of calculus replaces that of algebra: we deal with a continuous system instead of a discontinuous one.

Martin and Synge (616) have considered chromatography from this point of view. Since their treatment is applicable in principle to all distribution systems that meet the required assumptions, and since it leads to very fruitful operational conclusions, it is given here. This treatment deals explicitly with curves of type *A* (Fig. IV-1). Curves of type *B* require the use of more involved mathematics. The treatment may be visualized as extensions of those described in the above sections.

The physical system is a chromatographic column with a stationary (liquid) phase **S** and a mobile (liquid) phase **M** passing (downward) over it. (The words in parentheses are not meant as limitations, but as suggestions to aid visualization of the system.) Solute is distributed between the two phases.

In the column the mobile phase continually moves over the stationary, so that equilibrium is not established at any point in the column. However, the column can be divided in imagination and mathematically into layers of a thickness, h, such that the solution issuing from each layer is in equilibrium with the mean concentration of the solute in the nonmobile stationary phase throughout the layer.

"The behavior of a column consisting of a number of 'theoretical plates,' within each of which perfect equilibrium between the two phases occurs, can be described with great simplicity. Peters (724) showed that the continuous or packed type of distillation column (in which equilibrium is not established at any point) could be divided up into a number of layers each of which was equivalent to one theoretical plate; and the height of such a layer was called the H.E.T.P. or 'height equivalent to one theoretical plate.' For the present purpose the H.E.T.P. is defined as the thickness of the layer such that the solution issuing from it is in equilibrium with the mean concentration of solute in the nonmobile phase throughout the layer. It can be shown from diffusion arguments that the H.E.T.P. is a constant through a given column except when the ratio of the concentrations of the solution entering and leaving the plate differs

greatly from unity (849). It may be taken as constant for the chromatogram without serious error.

"For the equations to be manageable certain simplifying assumptions must be made, viz. that the diffusion of solute from one 'plate' to another must be negligible, and that at equilibrium the distribution ratio of one solute between the two phases must be independent both of the absolute value of its concentration and of the presence of other solutes.

"We consider here a chromatogram . . . having two liquid phases.' The extension of the theory to the usual adsorption chromatogram is obvious when the adsorption isotherm is a linear function of the concentration of solute in the liquid phase.

"Consider a chromatogram of many plates:

"Let h = the H.E.T.P.,

A = the area of cross-section of the column,

A_S = the area of cross-section of the non-mobile phase,

A_L = the area of cross-section of the mobile phase,

A_I = the area of cross-section of inert solid $(A_S + A_L + A_I = A)$,

v = the volume of solvent used in development of the chromatogram,

a = the partition coefficient, i.e.,

$$\frac{\text{g. solute per ml. of non-mobile phase}}{\text{g. solute per ml. of mobile phase}} \text{ at equilibrium,}$$

$$V = h(A_L + aA_S),$$

$$\mathrm{R} = \frac{\text{movement of position of maximum concentration of solute}}{\text{simultaneous movement of surface of developing fluid in empty part of tube above chromatogram column}}$$

r = serial number of plate, measured from top of column downwards,

Q_r = total quantity of solute in plate r.

"Consider the case where unit mass of a single solute is put into the first plate, and is then followed by pure solvent. We can draw up a table showing the quantity of solute in each plate after successive infinitesimal volumes of mobile phase δv have passed:

Vol. of solvent passed $(r\delta v)$	Serial number of plate $r = 1$	2	3	4	5
0	1	0	0	0	0
1	$(1 - \delta v/V)$	$\delta v/V$	0	0	0
2	$(1 - \delta v/V)^2$	$2(1 - \delta v/V)\delta v/V$	$(\delta v/V)^2$	0	0
3	$(1 - \delta v/V)^3$	$3(1 - \delta v/V)^2(\delta v/V)$	$3(1 - \delta v/V)(\delta v/V)^2$	$(\delta v/V)^3$	0
4	$(1 - \delta v/V)^4$	$4(1 - \delta v/V)^3(\delta v/V)$	$6(1 - \delta v/V)^2(\delta v/V)^2$	$4(1 - \delta v/V)(\delta v/V)^3$	$(\delta v/V)$

"We see that the quantity in each plate is a term of the binomial expansion $((1 - \delta v/V) + \delta v/V)^n$ so that when n successive volumes of solvents δv have passed,

$$Q_{r+1} = \frac{n!\,(1 - \delta v/V)^{n-r}\,(\delta v/V)^r}{r!\,(n-r)!}$$

Now when n is large, this becomes

$$Q_{r+1} = \frac{1}{r!}\,(n\delta v/V)^r e^{-n\delta v/V}$$

But $n\delta v$ is the volume of solvent that has been used to develop the chromatogram. Putting $n\delta v = v$,

$$Q_{r+1} = \frac{1}{r!}\,(v/V)^r e^{-v/V}$$

which, by Stirling's approximation, becomes

$$Q_{r+1} = \frac{1}{\sqrt{2\pi r}}(v/rV)^r e^{r-v/V} \tag{4}$$

when r is large (>10).

"Now Q_{r+1} is a maximum and equals $\dfrac{1}{\sqrt{2\pi r}}$ when $v/r = 1$, so that the position of maximum concentration has moved a distance hv/V directly proportional to the volume of liquid v which has flowed through. In terms of the movement of the surface liquid standing above the solid in the tube, the relative rate of movement $\mathbf{R}$ is given by the expression

$$\mathbf{R} = \frac{vh/V}{v/A}, \text{ i.e., } \frac{\text{movement of band}}{\text{movement of surface}}$$

$$= \frac{Ah}{V}$$

$$= \frac{A}{A_L + \alpha A_S} = \frac{A_L + A_S + A_I}{A_L + \alpha A_S}$$

or

$$\alpha = \frac{A}{\mathbf{R}A_S} - \frac{A_L}{A_S} \tag{5}$$

"If we plot the concentration of solute in plate $r + 1$ against v/V using equation (4) we obtain the curve shown in Fig. IV-10. When r is infinite

Fig. IV-10. (6161). Distribution curve. The points represent the relation between Q and v/V for $r = 100$, *cf.* equation (4). The full line is the normal curve of error with abscissa t, i.e., $Q/Q_{\max.} = \exp\,[-{}^1/_2 t^2]$.

this curve becomes the normal curve of error. When $r > 100$ the ordinates of the curve can be expressed in terms of the normal curve of error by

$$\frac{v}{V} = r + t\sqrt{r} + \frac{t^2}{3}$$

Similarly, the area under the curve can be expressed by

$$\frac{v}{V} = r + t\sqrt{r} + \frac{t^2}{4}$$

where t is the abscissa of the error curve.

"Characteristics of the curve for various values of t are shown below:

t	$Q/Q_{\max.}$	Area under tail of curve as % of whole area under curve
1	0.605	15.9
2	0.135	2.27
3	0.011	0.13

"Now if two solutes be present with partition coefficients α and β, then practically complete separation will be obtained when $t = 3$; then

$$\frac{A_L + \alpha A_S}{A_L + \beta A_S} = \frac{r - 3\sqrt{r} + 2.25}{r + 3\sqrt{r} + 2.25} \tag{5b}$$

since only <0.2% of the substance whose partition coefficient is β has passed the $(r + 1)$th plate and >99.8% of the other substance whose partition coefficient is α has passed it. Figure IV-11 shows graphically the relation between $\frac{A_L + \alpha A_S}{A_L + \beta A_S}$ and r for various values of t, i.e., for various degrees of separation.

"It follows from the assumption that the partition coefficient is a constant that there will be no interaction between various chromatograms occurring simultaneously in the same tube. Hence, if, as in the practical case, the whole of the solute was not initially in the first plate, but distributed over a number, then the concentration at any plate and time will

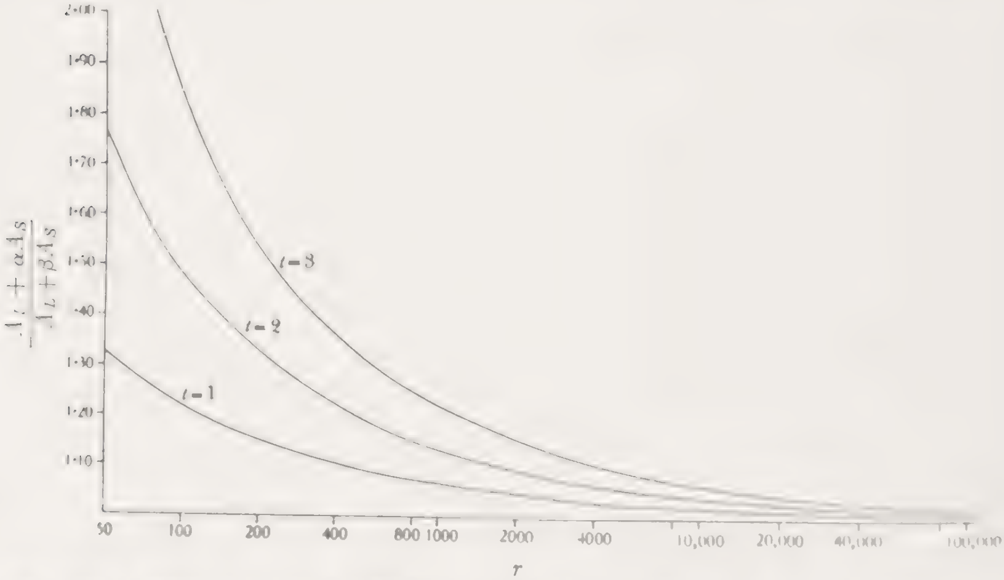


Fig. IV-11. Relation between difference in partition coefficient, number of plates (r) and degree of separation (t). *Cf.* equation (5b). (From Martin and Synge (616).)

be the sum of the concentrations for the relevant number of chromatograms started successively. Thus the region of maximum concentration will be broadened, and the total width of the band will be greater than that shown in Fig. IV-10 approximately by the initial width of the band before development by solvent was begun. The number of plates required for resolution will be correspondingly increased.

"The height equivalent to a theoretical plate depends upon the factors

controlling diffusion and upon the rate of flow of the liquid. There is an optimum rate of flow in any given case, since diffusion from plate to plate becomes relatively more important the slower the flow of liquid and tends of course always to increase the H.E.T.P. Apart from this, the H.E.T.P. is proportional to the rate of flow of liquid and to the square of the particle diameter. Thus the smallest H.E.T.P. should be obtainable by using very small particles and a high pressure difference across the length of the column. The H.E.T.P. depends also on the diffusibility of the solute in the solvent employed, and in the case of large molecules, such as proteins, this will result in serious decrease in efficiency as compared with solutes of molecular weights of the order of hundreds.

"The separations obtainable in practice are less than the theory predicts for two principal reasons. First, the partition coefficient is seldom a constant, usually decreasing as the solution becomes stronger. This results in the front of the band becoming steeper, and the back flatter and, more importantly, in the band becoming wider, since the concentrated part moves faster than the dilute part. This effect can sometimes be diminished by working with initially dilute solutions.

"Interaction between two solutes often, however, leads to an increase in separation over the theoretical, the more strongly absorbed solute "eluting" the less strongly absorbed, and thus tending to cause a sharp boundary between the two, reminiscent of the behavior of ions in the moving boundary in transport number determinations.

"The other great source of loss of efficiency lies in lack of uniformity of flow through the column. This lack of uniformity often prevents good separations being realized even though the solutes be separated in the column itself, as the cut cannot follow the required surface. In striving for conditions for uniformity of flow, the high pressure and small particle size desirable for smallest H.E.T.P. have to be abandoned." For a treatment of zone holding-time distributions of the Gaussian type see Klinkenberg and Sjenitzer (481a).

VI. THEORY OF ADSORPTION

The previous section dealt explicitly with chromatograms in which the distribution follows the linear type of isotherm (A, in Fig. IV-1). In adsorption distributions the isotherms, as stated already, are most usually of the type B. Theory has been developed to deal with these cases, where α is not constant. Curves of the type C, being the inverse of B, are also dealt with. For example, the Freundlich equation (2), where $q = K\ C^n$, where it is applicable, yields B-type curves when the exponent is less than 1 ($n < 1$). Where $n = 1$, the curve is linear. Where $n > 1$, the curve is of the C type.

In many of the theoretical treatments of chromatography the Langmuir equation (519,520), because it is convenient, is used for *B* type adsorption in preference to the Freundlich equation. The Brunauer, Emmet, and Teller equation is also theoretically based (128), but neither this equation nor the theories of Zsigmondy (1042) or Polányi (329,737) seem to be much used. We barely touch the surface, here, of a tremendously complicated field of knowledge, more insight into which must be sought in specialized volumes (7,128).

The **Langmuir equation** was derived from the consideration that a gas molecule striking an impermeable and *perfectly homogeneous* solid surface may either rebound immediately or else condense for an appreciable period upon the surface. Langmuir also considered that in the cases under investigation by him the surface forces of attraction were short-range forces, so that absorbed films were only one molecule thick on the average. Under these limitations the rate of condensation of the adsorptive on the surface could be written as $a\theta\mu$. Here μ represents the amount of adsorptive molecules (gram moles) striking unit surface of the adsorbent per second; θ represents the fraction of surface which is bare and so available for condensation of adsorptive, the already covered part of the surface being considered to hold a second layer so feebly that a molecule striking it evaporates at a rate effectively equivalent to a reflection; a is a proportionality constant which is usually close to but never exceeds unity. Molecules evaporate from the surface at a rate $\nu_1\theta_1$, where θ_1 is the fraction of surface actually covered by adsorptive, and ν_1 is the rate of evaporation from a completely covered surface. At equilibrium the rate of condensation (adsorption) is equal to the rate of evaporation (desorption), whence:

$$a\theta\mu = \nu_1 \theta_1$$

since

$$\theta + \theta_1 = 1$$

then

$$\theta_1 = a\mu/(\nu_1 + a\mu)$$

By comparing this equation with an isotherm of Type I (Fig. IV-12), it is evident that, as in the isotherm, at low pressures the amount of surface covered (θ_1) will be nearly proportional to the pressure (μ is related to the pressure p thus:

$$\mu = 43.75 \times 10^{-6}p/(MT)^{1/2}$$

where M is the molecular weight of the gas and T the absolute temperature). As the pressure increases θ_1 increases more slowly and finally the surface becomes saturated ($\theta_1 = 1$). The extent of adsorption is then in-

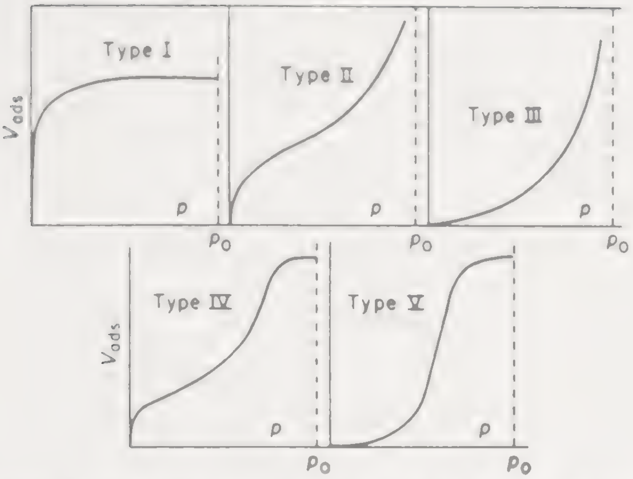


Fig. IV-12. Types of isotherms (129). Five types of van der Waals adsorption isotherms. Here V_{ads} is the volume of gas adsorbed per gram of adsorbent; p is the equilibrium pressure at which the adsorption has occurred; p_0 is the saturation vapor pressure of the adsorptive. These isotherms are found in the adsorption of gases and vapors on solids. An isotherm of Type I would belong to Type B in the simpler scheme of Fig. IV-1, and Type III to Type C.

dependent of the pressure. In a later paper Langmuir extended his calculations to surfaces with more than one kind of elementary space, and to various other types of surfaces and of behavior of adsorbed molecules, and extended it to films more than one molecule in thickness. The Langmuir equation has been thoroughly discussed by Brunauer, who has also given thermodynamic and some statistical derivations of the theory.

The Langmuir equation may be rewritten in various forms. The constants a and ν_1 may be combined:

$$\beta = a/\nu_1$$

then:

$$\theta_1 = \beta\mu/(1 + \beta\mu)$$

The fraction of surface covered, θ_1, is proportional to the number of moles of adsorptive absorbed divided by the area of the interface, or to the number of grams adsorbed (x) divided by the mass of adsorbent (m), assuming equal masses to have equal surface areas. Then, in the latter case:

$$\theta_1 = kx/m$$

As pointed out above, μ is proportional to the pressure, whence:

$$x/m = k_1k_2p/(1 + k_1p) \tag{6a}$$

Taking θ_1 as equal to the ratio of the volume of gas (v) removed by adsorption to the surface at a pressure p to the volume which would be adsorbed (v_m) if the surface were completely saturated with a unimolecular layer, that is:

$$\theta_1 p = v/v_m$$

then:

$$v = v_m Bp/(1 + Bp) \tag{7a}$$

or

$$p/v = (1/v_m B) + (p/v_m) \tag{7b}$$

If in equation (6a) $p/(x/m)$ is plotted against p, or in equation (7b) p/v is plotted against p, straight lines should be obtained. Brunauer (128) has emphasized that the constants B and v_m are neither arbitrary nor empirical and that for the Langmuir equation to be considered applicable to a given set of data not only must the straight-line plot be obtained but also the evaluation of B and v_m from the slope and intercept of the line must yield reasonable values. It may be noted that the area of the absorbent could be obtained from v_m if the cross-sectional area of the adsorbed molecule were known, and the monolayer were tightly packed.

Tiselius (933) has utilized the Langmuir equation to clarify the behavior of eluents, displacers, and saturators in chromatographic processes. Writing the equation in the form:

$$q = k_1 k_2 c/(1 + k_1 c) \tag{6b}$$

where q represents specific adsorption (x/m), c is equilibrium concentration, and k_1 and k_2 have the same significance as in equation (6a), but different values, he points out that it appears that saturators, which in small amounts block off the more active sites on the adsorbent surface, displacing the zone adsorptive, act to modify the constant k_2. They decrease the amount of adsorptive, that might be taken up at saturation, but do not make the isotherm less curved. Eluents affect k_1, and tend to make the isotherm less curved (see Chapter VIII, Section IX, B(2)).

It may be noted here for later recall that the Langmuir equation (6b) is sometimes written, when referring to adsorption from solution, as:

$$q = f\ (c) = a\ c/(1 + b\ c) \tag{8}$$

Here q represents specific adsorption, c is the equilibrium concentration, and the constants are gathered in a and b. At very high concentrations, q has a finite upper limit, a/b, which gives the amount of adsorptive adsorbed at saturation per gram of adsorbent. At sufficiently low concentration, where bc is much less than 1, the equation approaches the linear isotherm.

$$q = ac \tag{9}$$

Also, since

$$dq/dc = a/(1 + bc)^2 \tag{10}$$

the slope is a decreasing function of c, with a finite value a where $c \to 0$. Thus, a is the initial slope of the isotherm, the adsorption coefficient, the dimension of which is volumes per gram.

It may be noted, also, that where adsorption is weak, the isotherm will tend to be less curved than when it is strong. This is because the curvature is a function of the ratio of effectively available spaces to the concentration of molecules available to fill them. At low concentrations there is more space for adsorption than at high, where the surface is well filled by a strongly adsorbed substance. Also at low concentrations there are fewer molecules to be adsorbed. Thus all isotherms of the Langmuir type tend to linearity at low concentrations. When the molecules of adsorptive are weakly held, these considerations extend into higher concentration regions, and this phenomenon is utilized by Alm and coworkers (13) in connection with gradient elution (see below).

The Freundlich type of isotherm, however, reflects an additional phenomenon (which may also be present to an extent in systems that are fitted by the Langmuir isotherm). This is an inhomogeneity of the surface such that strong initial adsorption occurs specifically to more active sites on the adsorbent, the surface of which is inhomogeneous.

A very great advance in the direction of a general theory of physical adsorption was made by Brunauer, Emmett, Teller, Deming, and Deming (129,130). First they generalized the treatment of Langmuir and were able successfully to account for the three types of isotherms, I, II, and III (Fig. IV-12) of five that have been observed. In a later paper a still more general equation was derived which was adequate to describe satisfactorily the shapes of all five isotherms throughout the range of adsorption, as well as to calculate the effects of temperature change upon the adsorptions. Detailed discussion of this does not belong here (52,186, 229,323–328,330,381,854,855,918–920,966,978,984,1010).

VII. ADSORPTION CHROMATOGRAPHY

The quantitative theory of adsorption chromatography was first tackled by Wilson (1010), and has been extended by many other investigators. The approach described in this section assumes equilibrium between adsorbent and adsorptive, and neglects diffusion along the column. I am indebted to Professor Henry C. Thomas for the material in this section; it amounts to a summary of previous work. This is not a full treatment of the problem. That does not seem to belong here.

The chromatographic column is taken to be uniform in cross section, the developer is taken to flow at a constant velocity through the adsorbent, and the latter is taken to be uniformly packed. Terms are defined for convenience as follows:

- c_i Millimoles of ith component per milliliter of the solution (concentration in the mobile phase).
- q_i Millimoles of the ith component on the surface of 1 g. of the adsorbent: the specific adsorption. (This is also the concentration in the stationary phase.)
- s Fractional free space (interstitial volume) in milliliters per gram of material in the column.
- t Time.
- v Volume of liquid that passes into the top of the bed of adsorbent.
- $\dot{v}$ Flow rate in milliliters per minute. This is taken as a constant. In an actual system there will be great differences in velocity across a front of moving developer as it passes between particles of different shapes further apart or closer together. The velocity over the moving front is an average value.
- x "Length" of the column down to a certain level measured in grams. This is convertible to centimeters if the column is uniformly packed and the grams of adsorbent per centimeter is known.
- y Volume of liquid downstream from the level x. $y = v - sx$.

Diffusion is an extremely important factor in the chromatographic process. The rate is proportional to gradient in concentration, and there are, at fronts, for example, rather steep gradients. Furthermore, it seems that if a solute were introduced as a layer at the top of a bed of inert material and were washed down through it, the concentration profile of the zone would become that of a probability distribution (see Fig. IV-5) *under the influence of diffusion alone* (243). Nevertheless, the effects of diffusion will be neglected. Also, equilibrium will be assumed, though there will not, in the usual chromatographic analysis, be any overall equilibrium between adsorptive and adsorbent, owing to the differential countercurrent nature of the process (though there may be a kind of local steady state at certain places in the column).

Considering what happens with a single component i, in the small distance dx in a column (Fig. IV-13,*a*), one equates, to conserve mass, what passes into this layer to what remains in the layer in the interstitial fluid and on the adsorbent, plus what passes out of the layer. The fluid carrier, the solvent, or developer, is treated throughout as a constant factor.

Material that enters the layer is $c\delta v = c_i\dot{v}\delta t$. Here the notation δ implies that we have some control over the quantity. That is, we can

push more or less liquid through this layer comprised by dx. What remains in the interstices between the particles of adsorbent is $dx\, S(\partial c_i/\partial t)\delta t$. The amount adsorbed is $dx\,(\partial q_i/\partial t)\delta t$. (To take diffusion into account another term would have to be included to describe what has diffused downstream.) The amount that runs out of the layer is

$$[c_i + (\partial c_i/\partial x)dx]\dot{v}\delta t$$

Then

$$c_i\dot{v}\delta t = dx(\partial q_i/\partial t)\delta t + dxS(\partial c_i/\partial t)\delta t + [c_i + (\partial c_i/\partial x)dx]\dot{v}\delta t \qquad (11)$$

This simplifies to the following conservation equation:

$$(\partial q_i/\partial t)_x + S(\partial c_i/\partial t)_x + \dot{v}(\partial c/\partial x)_t = 0 \qquad (12)$$

Into this equation are now introduced the natural variables, $x = x$, the adsorbent upstream, and $y = \dot{v}t - Sx$, the fluid downstream. The equation becomes (229,326,984,1010):

$$(\partial q_i/\partial y)_x + (\partial c_i/\partial x)_y = 0$$

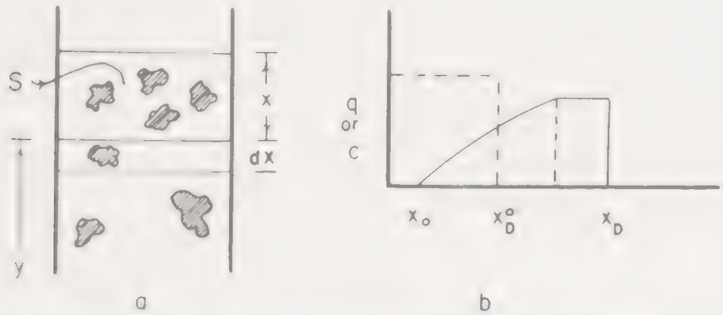


Fig. IV-13. Meaning of terms: (*a*) represents schematically a section through a column. The cross-hatched areas are particles of adsorbent with interstices, S, between them. Here x is the length of the column down to small section dx, in grams, and y is the volume of liquid downstream from dx. (*b*) relates the terms indicated in the text.

or, for S small,

$$(\partial c_i/\partial x) + \partial q_i/\partial v = 0$$

There is one such equation for each component, but it is not useful as it stands because it contains two dependent and two independent variables, so that additional information must be introduced, and further simplifying manipulations.

A function F is introduced, as permitted by the fact that in the above equation c and q are differentiable functions of x and y, such that:

$$dF_i = q_i dx - c_i dy \qquad (13)$$

This yields another form of the conservation equation:

$$F_i = \int (q_i \mathrm{d}x - c_i \mathrm{d}y)$$

Here, F_i is the total amount of solute i in the column. This equation says in essence that what has happened in the column can be determined at any given instant by integrating q over the column for increasing x and c for increasing y:

$$q = \partial F/\partial x$$
$$c = -\partial F/\partial y$$

Since equilibrium is assumed, t need not be retained in the conservation equation.

The adsorption equation is taken as

$$q = f(c)$$

With this additional information, the conservation equation becomes:

$$(\partial c/\partial x)_v = -[S + f'(c)](\partial c/\partial v)_x$$

This is altered algebraically to the experimentally more convenient form, as indicated by deVault (229):

$$\mathrm{d}x/\mathrm{d}v = 1/[S + f'(c)] \tag{14}$$

This is the rate of advance of a level in the column at which a fixed c is found. In the region of varying concentration the movement of a zone is given by

$$x = v/[S + f'(c)]$$

One has to know how $f'(c)$, that is, the first derivative of q with respect to c, varies with c. In the ordinary type of adsorption isotherm (Curve B, Fig. IV-1; curve I, Fig. IV-12) $f'(c)$ decreases as c increases. According to the equation, for smaller values of $f'(c)$ larger values of dx/dv are obtained, and according to deVault, this is the condition for a sharp discontinuity in concentration at the front of the zone. The position of this sharp front is given by

$$x_D = x_D^0 + vc^0/[Sc^0 + f(c^0)]$$
$$= (v + v^0)c^0/[Sc^0 + f(c^0)]$$

(See Fig. IV-13A.) It will be noted that in the formation of the initial zone,

$$v^0c^0 = Sx_Dc^0 + x_Dq^0$$

and thus

$$x_D^0 = v^0c^0/(Sc^0 + q^0)$$

The various additional points of Fig. IV-12*b* are given by (see Glueckauf (324)):

$$x_v = v/[S + f'(c^0)]$$

and

$$x_0 = v/[S + f'(0)]$$

For a Freundlich isotherm, with $n<1$, $f'(0) = \infty$, and $x_0 = 0$; i.e., complete elution is everywhere impossible. The establishment of the point x_L^0 becomes important in an inverse way in frontal analysis. Here x_D^0 is given as well as c^0, and v^0 is the quantity to be found (see below).

For the separation of two substances, continuing to neglect diffusion and to assume equilibrium, and taking the adsorption of the solvent as a constant factor, two conservation equations may be written, one for each substance, 1 and 2.

$$\partial c_1/\partial x + \partial q_1/\partial y = 0$$

where $q_1 = f_1\ (c_1, c_2)$

$$\partial c_2/\partial x + \partial q_2/\partial y = 0$$

where $q_2 = f_2\ (c_1, c_2)$

It is only if we know the *mixed* adsorption isotherms that we can write the conservation equations for this case (13,475–477). These equations, when solved, apply only to the limiting case, where diffusion, nonequilibrium, and solvent effects can be neglected:

$$\partial c_1/\partial x + f_{11}(\partial c_1/\partial y) + f_{12}(\partial c_2/\partial y) = 0 \qquad (15a)$$

$$\partial c_2/\partial x + f_{21}(\partial c_1/\partial y) + f_{22}(\partial c_2/\partial y) = 0 \qquad (15b)$$

Here f_{11} is the partial of f_1 with respect to c_1, f_{12} is the partial of f_1 with respect to c_2, etc.

However, there are only two possibilities. Either the two concentrations in the column bear some functional relationship to each other, that is,

$$c_2 = \phi(c_1) \qquad (16)$$

or else there is no functional relationship: $c_1 = c_1\ (x,y)$; $c_2 = c_2\ (x,y)$. The former case is more interesting and important, and was treated by Glueckauf. He used the relation (16) to eliminate c_2 from the conservation equations. Following various manipulations he obtained from equations (15) and (16) the new conservation equation in the somewhat disguised simplified form:

$$-1/\phi' = [f_{11} + f_{12}\phi')/[f_{21} + f_{22}\phi'] \qquad (17)$$

This is a quadratic equation in ϕ' and is first-order and second-degree in ϕ.

For adsorptions described by the Langmuir isotherm, where for two components

$$q_1 = f_1(c_1c_2) = a_1c_1/(1 + a_1b_1c_1 + a_2b_2c_2) \tag{18a}$$

$$q_2 = f_2(c_1c_2) = a_2c_2/(1 + a_1b_1c_1 + a_2b_2c_2) \tag{18b}$$

equation (17) simplifies to:

$$b_1c_1 = \lambda b_2c_2 - (\lambda\Delta)/(1 + \lambda) \tag{19}$$

Here $\Delta = (a_2 - a_1)/a_1a_2$ and is a measure of the difference in adsorbabilities of the two substances, which can be measured (a_2 is taken greater than a_1). The constant of integration λ is not known. In effect it determines the slope of the line obtained by plotting c_1 against c_2. In solving the quadratic (19) for λ, using for c_1 and c_2 two coexisting concentrations such as the initial concentrations, one obtains two values. Then two values of ϕ' are obtained in the relation $1/\lambda = (a_1b_2/a_2b_1)\phi'$. With one of these, a meaningful result is obtained. This is put into the conservation equation (19) and leads to the solution of the problem.

Calculations based on these equations lead to the picture of a zone with a sharp front, with the trailing portion of the zone (the "tail") starting quite sharply from the main body of the zone. Figure IV-14 shows the expected concentration profiles during a separation of two substances. Substance 2 is more strongly adsorbed than substance 1, and therefore displaces it even in the initial mixed zone (a, Fig. IV-14). This displacing effect extends to the tail of the leading zone (substance 1) so that this is actually sharpened (Fig. IV-14,b). Figure IV-14,c, shows a separation that is just complete. In practice, the development would be continued until there is an essentially empty region of adsorbent between the zones of components 1 and 2. The length of column necessary for complete separation (**X**) is:

$$\mathbf{X} = m_2b_2(1 + \lambda + a_2\Delta)/a_1a_2\Delta^2 \tag{20}$$

where m_2 is the mass of adsorptive 2 (component, or substance, 2) applied to the column. For simplicity, the effect of interstitial solute is neglected. If it were taken into account, **X** would have a somewhat smaller value.

Conclusions from the simpler theoretical treatment have been summarized by Coates and Glueckauf (186). They point out that when the solutes follow a Langmuir type of isotherm separations are made easier by applying the initial zone from solutions concentrated enough to saturate the adsorbent. Under such initial conditions, a rough value for the

minimum amount (X_0) of adsorbent required completely to separate two solutes (1 + 2) can be obtained from

$$X_0 = L^0/(\Delta x_1/\Delta x_2 - 1)^2 \qquad (21)$$

Here L^0 is the amount (length of column) of adsorbent required to hold the initial zone, and Δx_1 and Δx_2 are the values for the distances moved by the rear boundaries of the zones of the single substances produced

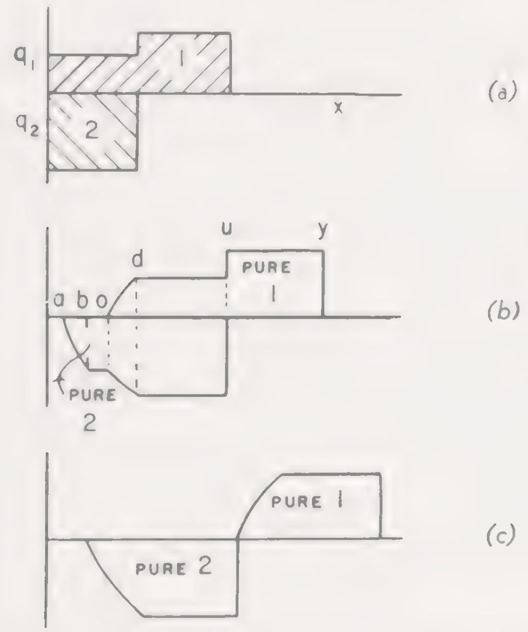


Fig. IV-14. Progress of a separation. (*a*) Profile of initial mixed zone of substances 1 and 2. Substance 2 is more strongly adsorbed than 1 and has displaced some of it. (*b*) Partial separation, some pure 1 and 2 present. (*c*) Separation just complete. The substances have isotherms of the Type I in Fig. IV-12. (After Glueckauf (324).)

under the influence of a given amount of developer. The values Δx_1 and Δx_2 are measured with the pure substances if they are available. Then for a good separation of the zones somewhat more than X_0 adsorbent would be used. They say, further, that with difficultly separable substances it is of advantage to add to the developer a third substance that is more strongly adsorbed than 1 or 2. The concentration of this substance should be at least equal to that of the other solutes. Then, the amount of adsorbent needed is approximately

$$X_0 = L^0/(\Delta x_1/\Delta x_2 - 1) \qquad (22)$$

but the zones are not completely separated and a mixed zone remains.

The values of Δr_1 and Δr_2 are determined as above, without the presence of the third solute. More recent work can be traced through reference (327a).

Chromatography is a nonequilibrium process. One of the factors that operate against the attainment of equilibrium is the finite rate of the adsorption or other distribution process. The theories that have been developed to deal with this, and to include as far as possible the effects of other factors such as diffusion, reinforce the strong similarity between differential countercurrent processes of all kinds. They are not useful in the ordinary chromatographic separation because the necessary data, such as mixed isotherms of the substances being separated, are usually not available. This makes the theory useless for the ordinary occasions of chromatography. Nevertheless in some areas the theories have shown some utility (307,687,920,921,966). For example, various approaches have been taken to ion exchange phenomena: an equilibrium treatment; a treatment in terms of rates of exchange; a treatment analogous to that used in distillation-column problems; a treatment based on a Donnan-type distribution. These have led to clarification of the mechanisms of the processes. Such more specialized considerations do not belong here (see Chapter IX).

VIII. SIMPLE DERIVATIONS OF R AND R_F

Simpler derivations of R, or R_F, have been advanced. For example, Le Rosen in his studies of adsorption chromatography approached the meaning of the R value in the following way (542). When a molecule is absorbed, it remains essentially motionless on the adsorbent. It is only while the molecule is in the moving developer that it travels along (up or down) the column. Let T_M be the average time that the average molecule of a zone remains in the developer (mobile phase) and T_S the average time it remains (adsorbed) in the stationary phase. Then the fraction of time that the molecule remains in solution, and so moves, is $T_M/(T_M + T_S)$. During development, when the developer is moving at a constant velocity (which may be measured as V_c, the distance moved per unit of time by the meniscus of developer above the column in a tube of uniform diameter) the velocity of the zone down the column will be equal to the velocity of the developer (in the terms measured) multiplied by the fraction of time that the molecules of the zone substance dwell in the mobile phase. Here R is the ratio of the velocity of the zone to the velocity of the solvent; hence:

$$R = [T_M/(T_M + T_S)]V_c/V_c \qquad (23)$$

and

$$R = T_M/(T_M + T_S)$$

Thus R is the fractional time in the mobile phase.

A relation between R and a linear isotherm was pointed out, in this connection, by Le Rosen. Let the amount of substance adsorbed on adsorbent in equilibrium with one unit volume of solution of concentration c be A. Then $A = k\ (c)$. Assuming instantaneous equilibrium, if a solution of concentration c is pressed into a column so that a zone of substance is built up, comprising unit volume of mobile phase, with the front of the zone sharp, and the interstitial fluid always having the concentration c right up to the front (the situation in frontal analysis), then the total amount of substance in the zone, both adsorbed and in the interstitial

Fig. IV-15. **Simple derivation of R_F** (529). (O) Location of center of initial zone, point of origin. (x) Distance moved by zone; $x + y$, distance moved by front. P, Paper strip. See text.

solution, is $(k + 1)c$. In order to build up this zone, the mobile phase must move through $k + 1$ elements of volume while the zone builds up in 1. Then movement of zone/movement of mobile phase, $R = 1/(k + 1)$. (If the zones change in width on development, this relation holds only approximately.)

A similarly simple approach was advanced by Cremer and Müller (201) in connection with gas adsorption chromatography, and rewritten by Lederer and Lederer for paper chromatography (529). It may be stated as follows (Fig. IV-15). If x is the distance moved by the center of density of a zone (x is proportional to the time spent in the mobile phase) and if

$(x + y)$ is the distance moved by the front of developer in a paper strip or column, then y is proportional to the time spent in the stationary phase, because the solvent flowed onward the distance y while the molecules of the zone material remained on the average immobile. In equation (1) α was defined as $f(C_S^{\text{equil.}}/C_M^{\text{equil.}})$; hence here α is proportional to y/x, or

$$\alpha = f(y/x)$$

If the isotherm is of the type A, then $\alpha = K\,(y/x)$, where K is the ratio of the cross section of the mobile to the stationary phase (their relative volumes may be used in a sufficiently short section of the paper).

R_F is defined operationally as $x/(x + y)$ and K is A_M/A_S, whence

$$R_F = A_M/(A_M + \alpha A_S)$$

This same reasoning applies to LeRosen's treatment if the condition of a linear isotherm is met.

The condition of an essentially linear isotherm was utilized explicitly by Tiselius (927). In adsorption chromatography he developed a method for analyzing (by interferometry) solutions so dilute that the isotherms could be taken as essentially linear. This introduced an extremely profitable simplification of theory.

The discussion so far has dealt with chromatographic processes of the development and elution types, in which calculation of R or R_F values was one aim, and a full description of the entire behavior of these chromatograms was the goal.

The next sections discuss other types of chromatography: frontal analysis, displacement analysis, and gradient elution analysis.

IX. FRONTAL ANALYSIS

The method of frontal analysis is in general as follows. The solution to be examined is passed through a bed of adsorbent. If the components of the solution are held by the adsorbent, then at first pure solvent will issue from the bed. If the bed had been initially saturated with solvent then this liquid, comprising the interstitial volume of the bed, will precede that solvent derived from the solution by retardation of adsorptives. As the bed becomes saturated with the adsorptives, first the least strongly adsorbed substance will break through at the end of the bed, then the next more strongly adsorbed, and the next, and so on until eventually the bed becomes saturated with all the adsorptives and the solution breaks through at the same concentration as it had on entering the column (Chapter II).

The phenomenon of frontal analysis is found in the old method of capillary analysis, and in the common industrial processes of batchwise adsorption, decolorization, demineralization, and so on, where the process uses a bed through which the solution to be examined is run to a break-through point.

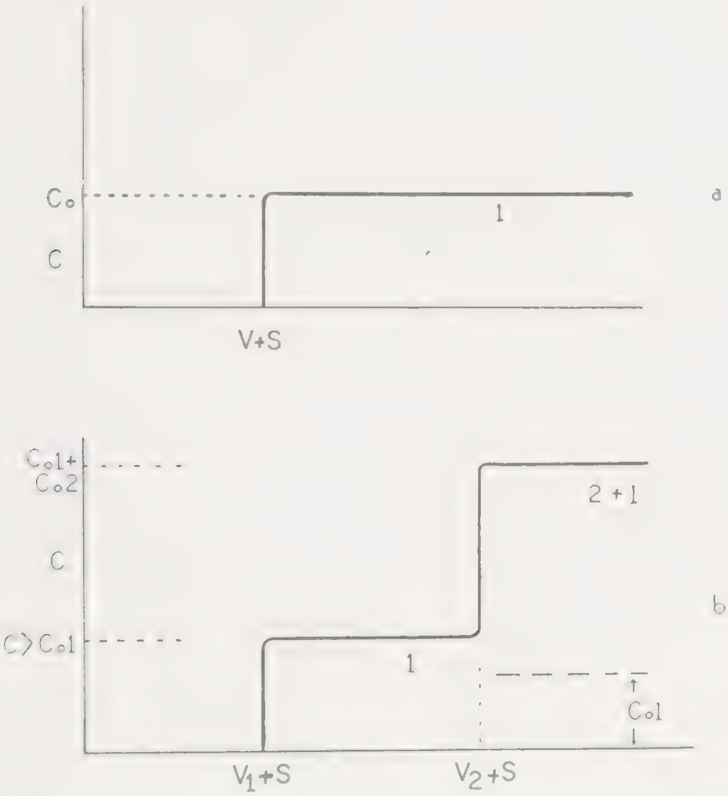


Fig. IV-16. Frontal analysis. For the meaning of the symbols see the text. The abscissa is marked off in units of volume, the ordinate in units of concentration, or some measurement such as refractive index that can be related to concentration. (*a*) A single substance, of concentration c_o, breaks through the column and forms a front at $v + s$. (*b*) A mixture of two substances in concentrations c_{o1} and c_{o2} forms two fronts: $v_1 + s$ and $v_2 + s$. (After Claesson (177).)

The theory of frontal analysis was worked out by Tiselius and Claesson (177,928,935). It describes the results of the method. With a solution containing a single solute a curve of the type shown in Fig. IV-16.*a* is obtained. The volume that appears up to the "step," the break-through "point" where the concentration of the effluent increases rapidly over a small volume, is called the retention volume. It is made up partly of the volume of liquid, if any, present in the interstices of the bed at the be-

ginning of the experiment (the bed is usually washed with solvent prior to the analysis so as to avoid heat-of-wetting effects); this is sm, where m is the total weight of adsorbent in grams, and s is the specific interstitial volume in milliliters per gram of adsorbent. The retention volume also includes the volume v of solution from which adsorptive was removed by the adsorbent, as well as the volume of any liquid in the "dead" space between the end of the adsorptive bed and the measuring instrument. The important volume that is to be measured is v, the *corrected retention volume*. When this is divided by the weight of adsorbent, the *specific* retention volume v^0 is obtained: $v^0 = v/m$. Sometimes it is convenient to measure v^0 in terms of s by dividing v^0 by s. This gives the number of s volumes required to break through and is dimensionless, hence applicable to columns of different sizes and amounts of adsorbent.

The amount of adsorptive retained by the *bed* of adsorbent is $vc_0 + smc_0$ where c_0 is the concentration of the solution applied to the column. The specific amount adsorbed is $q = v^0c_0$, and hence the total amount *adsorbed* is qm.

The ease with which q is measured by the frontal analysis technique for any given value of c_0 makes this one of the best ways of determining an isotherm: q is measured for several predetermined values of c_0 and the isotherm is then drawn, or equations of the Freundlich (2) or Langmuir (8) type are tested for fit by calculating the constants at the different values of q and c_0. This method is usually quicker and simpler than the method of shaking a known weight of adsorbent with a known volume of solution of c_0 and then after equilibrium is reached measuring the new, lower, concentration c, and calculating q from $(c_0 - c)/m$. In the frontal analysis method, equilibrium is indicated when the effluent has reached the same concentration as the applied liquid, namely c_0, and qm is calculated as the amount of adsorptive that has disappeared from the effluent. (See Chapter XV.)

When the frontal analysis is applied to a *mixture*, the more strongly adsorbed substances *displace* the less strongly adsorbed ones; hence the heights of the steps do not measure directly the concentrations of the substances in the original solution. Claesson has shown that when only two adsorptives are present in the mixture it is possible to obtain their isotherms in the presence of each other. The relation used is as follows:

$$\begin{aligned} &\text{adsorptive 1:} \quad q_1 = f_1(c_1,c_2) \\ &\text{adsorptive 2:} \quad q_2 = f_2(c_1,c_2) \end{aligned}$$

If v_1 is the corrected retention volume to the first step, and v_2 the corrected retention volume to the second (Fig. IV-16,*b*) then:

$$q_2 m = v_2 c_2$$

The v_2 of solution originally contained $v_2 c_1$ of adsorptive 1, of which the amount in step 1 was not adsorbed. This amount is $(v_2 - v_1)c_{1,1}$ where $c_{1,1}$ is the concentration of the first step. Then $q_1 m = v_2 c_1 - [(v_2 - v_1)c_{1,1}]$. Hence:

$$q_1 = f_1(c_1,c_2) = v_2^0 c_1 - (v^0_2 - v_1)c_{1,1} \tag{24}$$

$$q_2 = f_2(c_1,c_2) = v^0_2 c_2 \tag{25}$$

(These are the equations (12) and (13) of Claesson.)

The situation is more complicated with more than two adsorptives in the mixture. Claesson has given the theory for dealing with this situation in his monograph.

X. DISPLACEMENT ANALYSIS AND CARRIER DISPLACEMENT

Tiselius invented displacement analysis (930). The method in **adsorption chromatography** is usually applicable to the separation of mixtures of homologs, especially where the displacing agent is homologous with the components of the mixture. It requires that the distribution be reversible

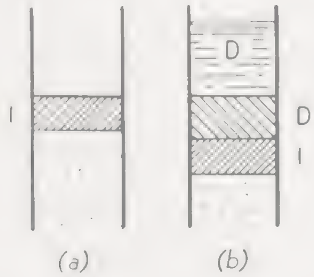


Fig. IV-17. Displacement (930). (*a*) A zone of substance 1 is applied to the column. (*b*) A displacer has been applied and has formed a zone *D*, pushing the zone of substance 1 ahead of it (177). See Fig. IV-16,*b*.

and that all the solutes be adsorbed on the same kinds of sites on the adsorbent, so that the more strongly adsorbed ones will displace less strongly adsorbed ones. The method can also be applied in partition chromatography, and an example in this field will also be given.

If a small amount of a substance 1 in solution is applied to a column of adsorbent previously wet with the solvent, the solute will be held as an initial zone with a sharp front, Fig. IV-17,*a*. In this chromatographic method, a solvent is chosen that is not strongly adsorbed, relative to the

solute, so that it acts merely as a carrier, and does not interfere appreciably with the formation of the initial zone.

If there is now applied to the column an excess of solution (in the same solvent) of a *displacing agent*, *D*, that is so strongly adsorbed that it expels substance 1 from the adsorbent, there will result the situation where

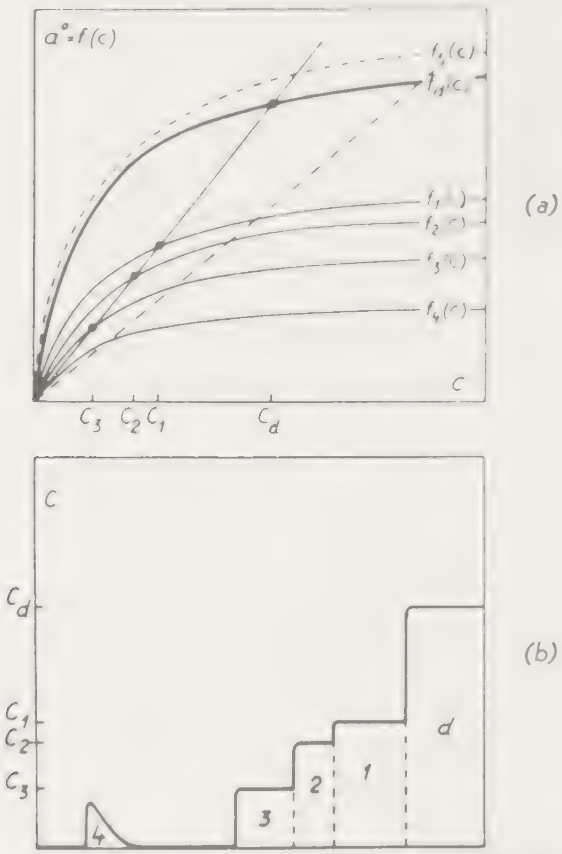


Fig. IV-18. Displacement analysis. (*a*) Adsorption isotherms showing theory of the displacement. Note that the equation of the straight line is $q = V_d{}^{\circ}C$, see text. (*b*) The zone of substance 4 is not displaced; substance 4 is such a poorly adsorbed substance that its zone runs ahead. Substance 3 is displaced by more strongly adsorbed 2; this by 1 and this by the displacer *d*. (After Tiselius (930).)

the displacer, acting like a "piston," forces ahead of its front, the compact zone of substance 1, as in Fig. IV-17,*b*. (If the substance 1 were so poorly adsorbed relative to the solvent alone that the solvent displaces it, then it will run on ahead of the front of developer as an isolated zone, "4," Fig. IV-18,*b*.)

After all the substance 1 has been displaced ahead of the substance *D*, then as more displacer is added, the velocity of movement of the front

of the lengthening zone of displacer and of the front of substance 1 will be the same. This velocity is related to the rate at which displacer solution is fed into the column; to the concentration of the displacer in the mobile phase; and to the concentration on the adsorbent (concentration in the stationary phase). For a displacer of fixed concentration c_D the specific equilibrium adsorption will take a specific value $q_D = f_D(c_D)$, where f_D is a constant. Now a certain volume v_D of displacer contains $v_D c_D$ of substance D, and will build up the zone of D by depositing part of this material on the adsorbent at a concentration q_D, and leaving some of the material in the zone interstitial fluid at a concentration c_D. Thus when Δv of displacer solution is forced into the column, the front of the zone of D moves a distance Δx_D. This distance is related to the volume and the concentration of the displacer:

$$\Delta v_D c_D = \Delta x_D m_0 f_D(c_D) + \Delta x_D s c_D$$

where m_0 is the grams of adsorbent per unit length of column. From this,

$$1/m_0[(\Delta v_D/\Delta x) - s] = f_D(c_D)/c_D$$

Then, under these conditions, where all of substance 1 is displaced ahead of substance D, and both fronts move with the same velocity as displacer is introduced into the column,

$$\frac{1}{m_0}\left(\frac{\Delta v}{\Delta x_D} - s\right) = \frac{f_D(c_D)}{c_D} = \frac{1}{m_0}\left(\frac{\Delta v}{\Delta x_1} - s\right) = \frac{f_1(c_1)}{c_1} \tag{26}$$

(This is Claesson's equation (42).)

This equation states that for a given concentration of displacer, after the steady state has been reached where the zone of 1 moves just ahead of the displacer, the ratio of $f_1(c_1)/c_1$, i.e., q_1/c_1, will take a fixed value equal to the ratio $f_D(c_D)/c_D$. This concentration will be independent of the *amount* of substance 1 in the initial zone. When the steady-state condition has been reached the zone of 1 will have adjusted its length so that it will accommodate all of the applied substance at this mobile phase/stationary-phase ratio.

Now if the initial zone contained a number of substances, each of which displaces the next (Fig. IV-18,*b*), the same considerations hold. The concentration of the most weakly adsorbed substance *that is displaced* (3) is set by that of the next (2), which is set by (1), which is set by that of the displacer, so that

$$\frac{f_D(c_D)}{c_D} = \frac{f_3(c_3)}{c_3} = \frac{f_2(c_2)}{c_2} = \frac{f_1(c_1)}{c_1} \tag{27}$$

Tiselius has given a graphical solution and illustration of this equation

as shown in Fig. IV-18,*a*. It is evident that if the isotherms of the substances are known, c for the substances can be calculated for any c_D. From the volume of the solution in any "step" of substance that issues from the column as this array of zones is forced out by the displacer, and the known concentration, the amount of that substance in the initial zone can be calculated.

Since an isotherm (Fig. IV-18,*a*) is characteristic of a substance, then it becomes possible to establish the concentrations c_1, c_2, c_3, c_n at which the substances 1, 2, 3, . . ., n will emerge from a column of a given adsorbent when they are displaced by a given displacer of fixed concentration c_D, whence in a mixture these can be recognized by their characteristic c values. Thus both qualitative and quantitative analysis is possible.

In the method of **carrier displacement analysis,** substances are introduced such that they form steps between the steps of the substances to be separated (see Chapter VIII). It is as if substance 2 were added to a mixture of 1 and 3 (Fig. IV-18,*b*) so as to space out their steps. Such carriers are chosen so as to be easily separable from the desired substances. Their function is to space out the zones so that any small "tails" that trail from a leading zone back into the following zone will have less chance of contaminating another desired substance. Thus a tail of 3 would be unlikely to extend through 2 into 1. Also, the use of an amount of carrier large compared to the zone carried enables one to work with quite minute amounts of initial mixtures. (See, however, Chapter VIII, Section VIII, 5.)

Displacement analysis is also applicable to **partition chromatography** as was stated in Chapter II. An example may be given substantially in the words of A. J. P. Martin (613).

The physical system is a column loaded with sodium hydroxide, or a buffer. Weak acids A, B, etc., have been displaced by another weak acid D, and the process of displacement development has reached a steady state (described above) where the zones of the components of the mixture move one ahead of the other in front of the advancing displacer (Fig. IV-18). It is assumed that the mobile phase contains un-ionized acid. Let:

"A^M = concentration of acid in mobile phase.
HA = concentration of unionized acid in stationary phase
A^- = concentration of ionized acid in stationary phase
$A = A^- + HA$ = concentration of total acid in stationary phase.
$a = HA/A^M$ = partition coefficient of unionized acid. Similarly for various forms of acids B and D, and buffer acids P_1, P_2 assumed insoluble in the mobile phase

H^+	=	concentration of hydrogen ions in stationary phase.
$K_A = H^+A^-/HA$	=	dissociation constant in acid.
M	=	cross-sectional area of mobile phase in column.
S	=	cross-sectional area of stationary phase in column.
T	=	cross-sectional area of total area of column.
R	=	ratio of rate of movement of zones divided by rate of movement of developing liquid in tube above column.
V_M	=	volume of mobile phase which passes a given zone in unit time.
V_S	=	volume of stationary phase which is passed by given zone in unit time $V_M/V_S = (T-RM)/RS$.
x	=	number of equivalent theoretical plates from top of column.
A_x	=	concentration of acid A in stationary phase leaving plate x.
$A^M{}_{(x-1)}$	=	concentration of acid A in mobile phase leaving plate $x - 1$).

"Considering first the equilibrium between acid in mobile and stationary phases we may write

$$\mathrm{Na}^+ + \mathrm{H}^+ = \mathrm{OH}^- + A^- + B^- + P_1^- + P_2^- + \ldots, \text{ etc.} \tag{28}$$

"If the concentration of the buffer is within practical limits and the acids not exceptionally strong or weak, H^+ and OH^- may be neglected; then

$$\mathrm{Na}^+ = K_A \frac{HA}{\mathrm{H}^+} + K_B \frac{HB}{\mathrm{H}^+} + K_P \frac{HP}{\mathrm{H}^+}, \ldots, \text{ etc.},$$

or

$$\mathrm{Na}^+ = \alpha K_A \frac{A^M}{\mathrm{H}^+} + \beta K_B \frac{B^M}{\mathrm{H}^+} + \frac{K_p P_1}{\mathrm{H}^+ + K_{p1}} + \frac{K_p P_2}{\mathrm{H}^+ + K_{p2}} \tag{29}$$

"Now consider a cross-section of the column, moving down the column at the same rate as the zones move. Since the column is in a steady state the amount of any of the substances forming the zone (i.e., any of the acids being separated, not those of the buffer), which is left behind in the stationary phase by the cross section as it moves, is equal to the amount of that substance which passes through the cross section in the opposite direction in the mobile phase. If the cross section is between theoretical plates $(x - 1)$ and (x) we have the material balance equation

$$V_S A_x = V_M A^M_{(x-1)} \tag{30}$$

and similar equations for each species of acid involved.

"Now in a region of constant composition such as the middle of zone A,

$$A^M_{(x-1)} = A^M_x = A^M_{(x+1)} \cdots, \text{etc.}$$

Hence

$$V_S A = V_M A^M,$$

$$\left.\begin{aligned} \frac{V_M}{V_S} &= \frac{A}{A_M} = \frac{\alpha(HA + A^-)}{HA} = \alpha\left(1 + \frac{K_A}{\mathrm{H}^+}\right). \\ \mathrm{H}^+ &= \frac{K_A}{V_M/\alpha V_S - 1} = \frac{\alpha K_A SR}{T - R(M + \alpha S)} \end{aligned}\right\} \tag{31}$$

i.e., the pH is determined by the R value and the partition coefficient and dissociation constant of the acid concerned, and is independent of the buffer. (R is, of course, a function of the buffer.)

"From equations (29) and (31), remembering that B^M is zero in band A, A^M may be calculated.

"Substituting D^M, the developing acid for A^M, the strength of developing acid to give a given R may be found, knowing the concentrations, dissociation constants of the buffer acids, the dissociation constant and partition coefficient δ of the displacing acid and the relative volumes of the phases in the column. From R and the dissociation and partition coefficients of displaced acids the pH and concentration in any zone of constant composition below may be found.

"In the special case of a NaOH column

$$R = \frac{T}{S(\mathrm{Na}^+/D^M + \delta) + M}\text{"} \tag{32}$$

Conditions in boundaries between zones may be found as follows. "Let us first define a layer across the column equivalent to a 'theoretical plate.' In such a layer the mobile phase leaving the bottom of the layer would be in equilibrium with the stationary phase at the top of it. A_x is in equilibrium with A^M_x. Similarly B_x is in equilibrium with B^M_x. (Strictly speaking A and B will have different diffusion constants, and the size of the equivalent theoretical plates will be different. This difference will be neglected and will not lead to any serious error.)

"These theoretical plates may be considered as stationary or as moving at any convenient speed in the column. We will let them keep pace with the zones, since then no change in composition will occur within any plate.

"Now by definition the liquids issuing from either side of the theoretical plate are in equilibrium. The ratio of A and B in the two phases can be found thus in plate x:

$$A_x^M = \frac{HA_x}{\alpha} = \frac{\mathrm{H}_x^+ A_x^-}{\alpha K_A} = \frac{\mathrm{H}^+}{\alpha K_A}\,(A_x^- - HA_x) = \frac{\mathrm{H}_x^+}{\alpha K_A}\,(A_x - \alpha A_x^M),$$

$$A_x^M = \frac{A_x \mathrm{H}_x^+}{\alpha K_A + \alpha \mathrm{H}^+}, \text{ similarly } B_x^M = \frac{B_x \mathrm{H}_x^+}{\beta K_B + \beta \mathrm{H}^+}$$

$$\frac{A_x^M}{B_x^M} = \frac{A_x\,\beta}{B_x \alpha}\left(\frac{K_B + \mathrm{H}_x^+}{K_A + \mathrm{H}_x^+}\right), \tag{33}$$

and H^+ may be found from equation (29) if both A_x^M and B_x^M be known. Now equation (30) in terms of ratios of A^M and B^M and A and B is

$$\frac{A_{(x-1)}^M}{A_x} = \frac{V_S}{V_M} = \frac{B_{(x-1)}^M}{B_x}, \quad \frac{A_{(x-1)}^M}{B_{(x-1)}^M} = \frac{A_x}{B_x}. \tag{34}$$

"Starting from any given concentrations of A_x^M and B_x^M we can in theory work from plate to plate and determine the concentration at any point in the column. Exact calculation is tedious, since H_x must be determined at each step. However, a good approximation is readily obtainable. Let us express equations (33) and (34) in logarithmic form. Let us plot them as in Fig. IV-19, where the vertical distance between the two lines is $\log \frac{\beta}{\alpha}\left(\frac{K_B + \mathrm{H}^+}{K_A + \mathrm{H}^+}\right)$. Now where $\log A/B < -2$, H^+ is that reigning in zone B. Where $\log A/B > 2$, H^+ is that reigning in zone A, and in those regions the curve will be parallel to the lower line expressing the material balance equation. The number of plates required to effect a given change in ratio of A^M/B^M is represented by the number of steps that can be drawn between the lines, each point on the upper curve representing conditions in one plate.

"It is obvious that replacing the centre sigmoid part of the curve by a straight line will give a good approximation so far as the number of steps is concerned, particularly in that the equations are symmetrical in A and B and $\frac{\beta}{\alpha}\left(\frac{K_B + \mathrm{H}^+}{K_A + \mathrm{H}^+}\right)$ will never differ much from unity in cases in which we are interested.

"From the argument above it is obvious that a good approximation will be given by.

$$\log\left(\frac{A_{(x+n)}^M}{B_{(x+n)}^M}\right) - \log\left(\frac{A_{(x-n)}^M}{B_{(x-n)}^M}\right) = n \log \frac{\beta}{\alpha}\left(\frac{K_B + \mathrm{H}_A^+}{K_A + \mathrm{H}_A^+}\right) + n \log \frac{\beta}{\alpha}\left(\frac{K_B + \mathrm{H}_B^+}{K_A + \mathrm{H}_B^+}\right)$$

If x is the plate where $A = B$, H_A^+ and H_B^+ are the hydrogen-ion concentrations in zones A and B, and $2n$ is the number of plates required.

"It should be noticed that the conditions in the column, and the mathematical treatment, are essentially similar to those in a distillation column run at total reflux.

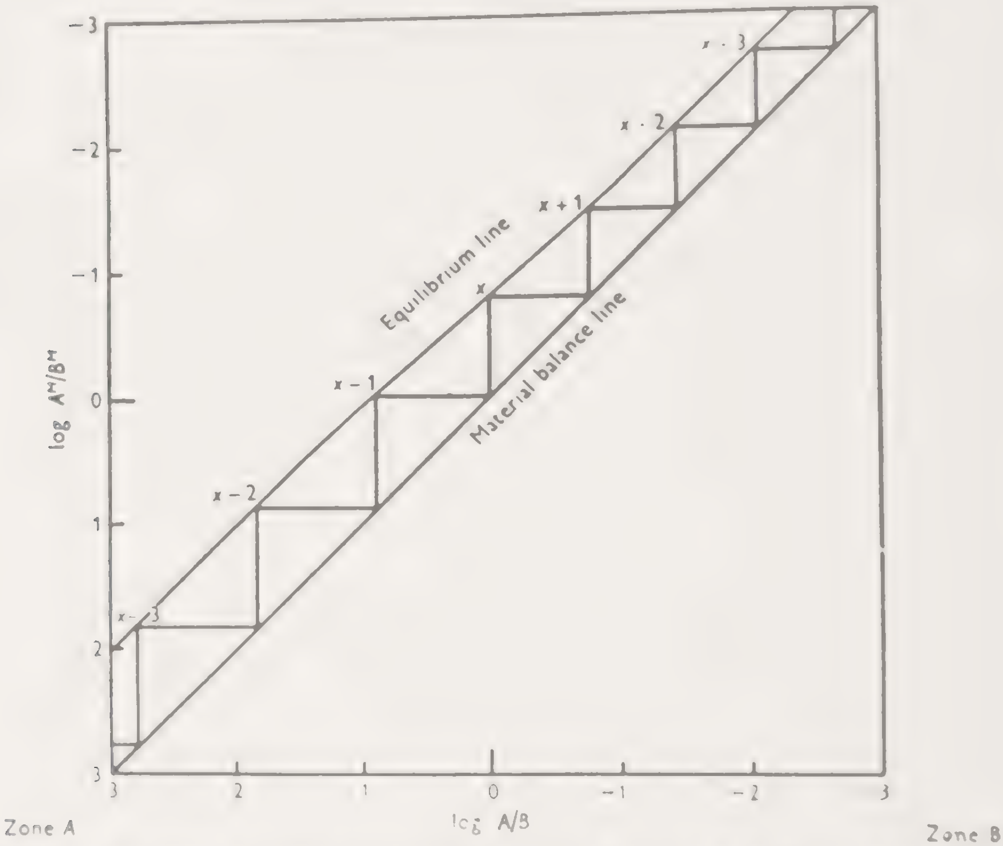


Fig. IV-19. Diagram showing relationship between number of theoretical plates and concentration in mobile and stationary phases. (From A. J. P. Martin (613).)

"Some examples of simple cases may be helpful here.

"Example 1. Consider a NaOH column. Let

$$K_A = K_B = 10^{-5},\ \alpha = 0.2,\ \beta = 0.1,\ \mathrm{Na}^+ = 0.1 \text{ normal } V_M/V_s = 1:$$

$$\mathrm{H}_A^+ = \frac{K_A}{V_M/\alpha V_s - 1} = \frac{10^{-5}}{5 - 1} = 2.5 \times 10^{-6},\ \mathrm{pH}_A \doteq 5.60,$$

$$\mathrm{H}_B^+ = \frac{K_B}{V_M/\alpha V_s - 1} = \frac{10^{-5}}{10 - 1} = 1.11 \times 10^{-6},\ \mathrm{pH}_B = 5.95.$$

Difference in pH in two zones = 0.35 unit. Concentration in zone A

$$A^M = \frac{Na^+H_A^+}{\alpha K_A} = 0.125 \text{ normal},$$

in zone B

$$B^M = 0.111 \text{ normal}$$

In this case, since $K_A = K_B$, the enrichment ratio $\frac{\beta}{\alpha}\left(\frac{K_B + H^+}{K_A + H^+}\right)$ is $\frac{\beta}{\alpha}$ and is independent of pH.

"The number of plates required to change the concentration ratio A^M/B^M from 0.001 to 1000 is

$$2n = \frac{\log\left(\frac{A^M_{x+n}}{B^M_{x+n}}\right) - \log\left(\frac{A^M_{x-n}}{B^M_{x-n}}\right)}{\log\frac{\beta}{\alpha}} = \frac{3+3}{0.301} = 20$$

Had a/β been 1.1 instead of 2.0, then 6/log 1.1 = 145 plates would have been needed.

"Example 2. NaOH column. Let

$$K_B = 10^{-5},\ K_A = 2 \times 10^{-5},\ \alpha = \beta = 1,\ Na^+ = 1 \text{ normal } V_M/V_s = 1$$

$$H_A^+ = 2.22 \times 10^{-6},\ H_B^+ = 1.11 \times 10^{-6},\ pH_A = 5.65,\ pH_B = 5.95,$$

$$\log\frac{\beta}{\alpha}\left(\frac{K_B + H^+}{K_A + H^+}\right) = -0.279 \text{ in zone } B$$

$$= -0.260 \text{ in zone } A,$$

$$A^M \text{ in zone } A = 0.111N = B^M \text{ in zone } B.$$

Number of plates required to change A^M/B^M from 0.001 to 1000

$$= \frac{2 \times 6}{0.279 + 0.260} = 22.$$

"Thus we see that for practically complete separation where the enrichment ratio is 2 only twenty plates are required. When it is 1.1 about 150 plates are required.

"Now it is difficult to separate substances differing in enrichment ratio by 1.1 by elution development, and though no figures are available for the height of the equivalent theoretical plate in buffered columns there

seems no reason to expect it to be greatly different from the water-laden silica columns."

The author further points out that complete separation in such systems is to be expected in a few centimeters of column.

"The distance which a displacement column must be run before a steady state is reached cannot be simply calculated. As the substances to be separated are first run into the column frontal analysis occurs. Though qualitatively it is easy to see what happens, the equations for three substances on a buffered column are troublesome to solve. While the transition to displacement development occurs as the developing acid flows in, the conditions become far more complicated, since some diffuse as well as sharp fronts will occur.

"A rough approximation may be made as follows. Assume that two of the substances A and B are much more difficult to separate than the others. When displacement development is complete let them occupy lengths a and b of the column and let the pH in their respective zones be pH_A and pH_B. Equation (31) may be written as

$$R_A = \frac{T}{M + \alpha S(1 + K_A/\mathrm{H}^+)}, \qquad R_B = \frac{T}{M + \beta S(1 + K_B/\mathrm{H}^+)}.$$

"If a value for H^+ be assumed equal to $(1/2)(\mathrm{H}^+_A + \mathrm{H}^+_B)$ (which should be a fair average), then the distance of the front of A from the top of the column should be approximately $\dfrac{a + b}{R_B - R_A}$ plus the width of any zones slower than A.

"The question may now be raised as to optimum pH to aim for to get sharpest separation and the relative advantages of using NaOH or buffer columns.

When $\beta/\alpha < 1$ and $K_B/K_A < 1$, a high pH is desirable.

When $\beta/\alpha < K_A/K_B < 1$, a low pH is desirable.

When $K_A/K_B < \beta/\alpha < 1$, a high pH is desirable.

"In all cases better separations can be expected with NaOH than with buffer, since larger pH changes occur down the column. (When the relative proportion of buffer to A and B, etc., becomes large, elution development rather than displacement development will occur. We shall not discuss the relative efficiencies of separation by these two methods.)

"It should be noted that buffer offers little advantage in choosing the pH at which the column should run. A suitable ratio of concentration of developing acid and NaOH can select any desired pH for a given zone.

"A buffered column may offer advantages in the detection of the passage of fronts, since a rise of concentration in the mobile phase can usually be expected as each front passes. With NaOH columns, these changes in concentration may be positive or negative (they depend solely on α, β, etc., not on the dissociation constant.)

"On the other hand, if pH changes be used to detect the passage of the fronts, NaOH columns will be the more sensitive. A 10% change in partition coefficient or dissociation constant should give rise to a change of about 0.04 pH unit, which should be detectable with suitable apparatus.

"Using conductivity changes in the stationary phase a buffer column may be better if there is a big difference between the mobilities of the buffer ions and the ions of the acids to be separated."

XI. GRADIENT ELUTION ANALYSIS

Gradient elution analysis was described by Alm, R. J. P. Williams, and Tiselius (13) as a method for minimizing some of the undesirable features of elution and development analysis, especially the tailing that occurs during the development of a zone of a substance which is characterized by a very curved isotherm. This was an explicit naming of a method that had been explicitly used for some time (645).

Essentially, the procedure consists in applying a zone of the mixture to be separated to a column of adsorbent, and then developing it with a liquid mixture the composition of which is continually changed in the direction which decreases the adsorption of the components of the mixture (933,12,239,533,607,645,1002). This continuous change is accomplished by adding an eluting agent continuously to a mixing chamber initially filled with the starting developer, at a rate such that as developer mixture flows into the column, it is replaced in the mixing chamber by eluent. As developer is fed out of the chamber, and eluent mixed into what remains, the concentration of eluent increases, and the liquid fed to the column steadily rises in eluent concentration or, stated more generally in desorbing power.

According to Alm and co-workers this procedure, in the best cases, causes the isotherms of the adsorbed substances to approach linearity as the amount of eluent increases, and so causes the zones of substance to move as well-defined regions, with the increasing gradient of eluent tending to minimize tailing through its displacing action. A further improvement in the results is achieved, they report, by loading the adsorbent with some substance that saturates the more active adsorption sites, when tailing is still further decreased (Chapter VIII, Section VIII).

The method was applied by Donaldson and Marshall and co-workers to separate organic acids, using a partition-type column, in which, however, the results seemed not to be entirely explainable on a liquid–liquid distribution basis (239,607). They give methods for calculating the concentration of eluent at given stages, the position of zones to the lengths of columns needed for given separations when a known procedure is scaled upward or downward in size (176).

The gradients referred to above may be concentration gradients employing miscible liquids. Many other kinds of gradients have been intentionally used in chromatographic analysis. Thus Mitchell, Gordon, and Haskins (645) employed a gradient in salt concentration to partially resolve enzymes on a chromatopile. The development was started with a buffered, strong solution of ammonium sulfate, which was diluted continuously with water. Gradients in hydrogen ion concentration (1003) have been used in the separation of inorganic substances, and of alkaloids (157). Williams has combined gradient elution with a temperature gradient in the column (1002). The temperature decreased from the top to the bottom of the column (with increasing retardation of the zone under this effect) and superimposed upon this was the gradient in eluting power, decreasing from top to bottom of the column. The combined effect, in an experimental run, yielded a better defined zone than gradient elution alone at a constant temperature.

In all these studies of mixed solvents it is desirable to recall the observations made by many investigators of adsorption, that very frequently adsorption from a mixed solvent goes through a minimum as the concentration of one component of the mixture is increased at the expense of the other (Chapter VIII).

XII. CONCLUDING REMARKS

The general theory of chromatography, a brief survey of parts of which has been given, aims at describing the course of a separation in terms of the appropriate concentrations, amounts of mobile and stationary phases, and of the distribution coefficients of the components of the mixture. However, when new separation problems are faced many of the above parameters remain unknown until after the chromatography has been carried out, when usually any interest in them is likely to be small. Hence in the ordinary course of events the general theory is not at present used to engineer separations.

Under the usual conditions of practical work the useful quantities that describe a separation are the operationally defined parameters R (or R_F and derived values) and the interstitial volume of the column

and weight of adsorbent. The latter are useful for calculating from one column to another, and the R values are the most important observed quantities, being dimensionless, and characteristic of the substance in a given system of mobile and stationary phases.

General theory is useful chiefly because it gives the anatomy of R or R_F values, and so lends a framework for the classification of the factors that affect R or R_F (for example, see Chapter VII).

There is, however, another kind of general theory—also in a sense a part of the theory of chromatography—which is potentially more useful to the chromatographer. This is the theory that connects the R or R_F value of a substance in a given system with the chemical structure of the substance. This general theory transcends chromatography in that it is applicable to all distribution processes. It connects the distribution coefficient α with chemical structure, usually in terms of functional groups.

The R value, then, stands in a sense between two theories. One that has been sketched in this chapter, connects it with the physical course of the chromatography, and is not very helpful in new problems except as it gives a conceptual framework for organizing the factors that operate in the separation. The other connects it to the chemical properties of the components of the mixture, and even in its present poorly developed state is very helpful in solving new separation problems because it can suggest, on the basis of observed behavior of the zones, what to do to change the R values in a desired direction. This theory is taken up in Chapter XIII.

CHAPTER V

Gas–Liquid Partition Chromatography (Vapor–Liquid Partition Chromatography)

I. PRINCIPLE

The mixture is separated using chromatographic distribution between a moving *gas or vapor* phase (the developer) and a stationary liquid phase. Retardation of components results through interactions with the stationary phase. This method is developing so rapidly and becoming so specialized in its ramifications (materials, apparatus, special techniques) that its growth resembles that of the early period of paper chromatography. Only the fundamentals of the method and a brief survey will be given here (469b,730a,730b).

II. APPARATUS AND MATERIALS

The chromatographic column consists of a glass tube contained in a heating jacket, and packed with the stationary phase supported on a porous, inert material. Gas is fed to the column at a controlled pressure. The effluent is analyzed for the components of the mixture that is separated, and for velocity of flow of the developer gas. (See Figs. V-1,2.)

James and Martin (441) have described a simple apparatus. The chromatography tube is a length of 4 mm. I.D. glass tubing which has been drawn down at one end to a short length of thick-walled capillary. A small wad of glass wool is pushed down to the capillary to keep it from being blocked by the column material. The tube is packed as described below.

The packing that composes the chromatography column comprises a nonvolatile stationary phase mixed with an inert support (Table V-1). For example, to separate the lower fatty acids James and Martin dissolved 10% by weight of pure stearic acid in DC 550 silicone. This was intimately mixed with graded Celite in the ratio 0.5 g. liquid to 1 g. Celite.

The graded support (kieselguhr, diatomaceous earth) was prepared by suspending Celite 545 in water in a beaker 18 cm. high and decanting all material that had not settled in 3 min. After this grading process had been repeated several times the coarse material so obtained was heated in a muffle furnace at 300°C. for 3 hr. (presumably to remove organic

matter). The cooled material was leached with concentrated hydrochloric acid to remove iron and alkaline materials, and then thoroughly washed with water and dried at 145°C. Presumably any inert porous material that is wetted by a stationary phase can be used as a support in this method.

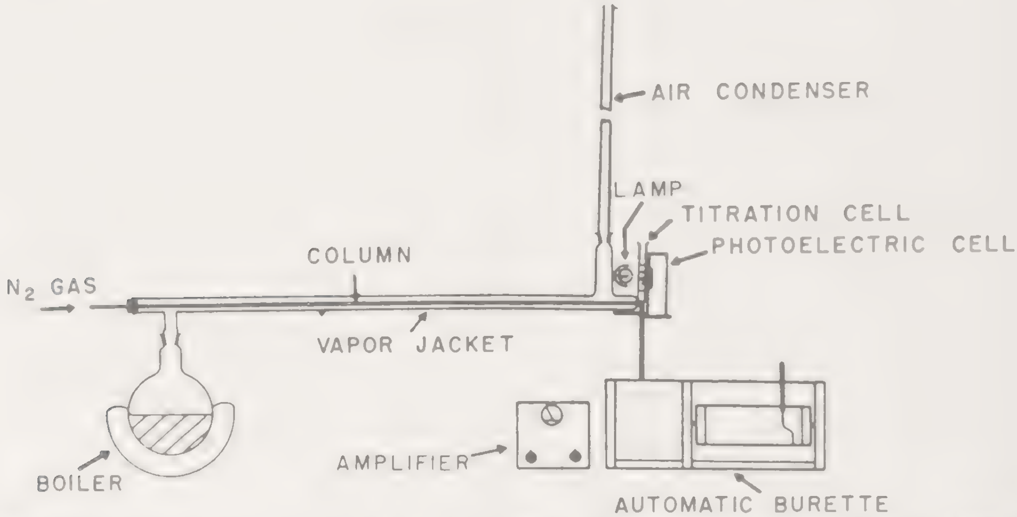


Fig. V-1. Schematic layout of a gas partition chromatography apparatus. (After James and Martin (442).)

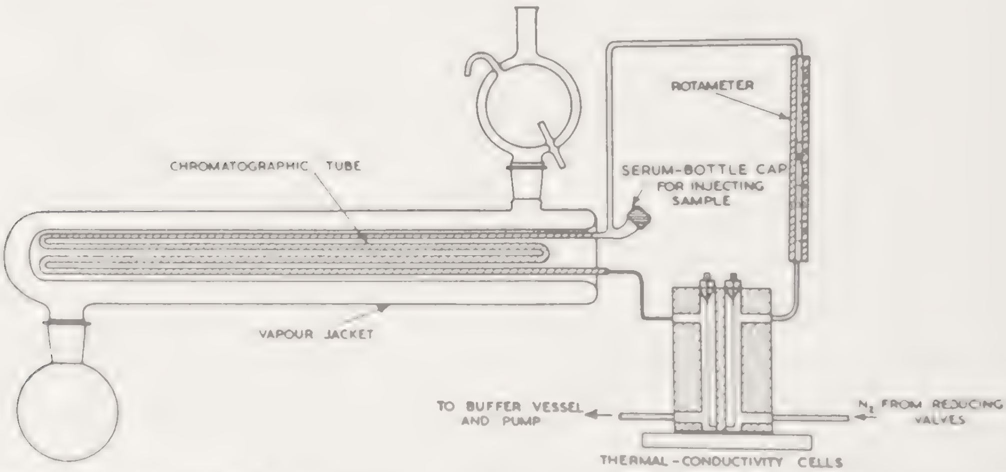


Fig. V-2. Layout of a folded column and accessories. Besides being folded (562) the column may be coiled (232a,247a,257a). (After Ray (765).)

The chromatography tube is packed to form a column according to the directions of James and Martin (441): "A small funnel is attached to the tube by a short length of pressure tubing and is then half filled with

TABLE V-1

Compilation of Some Data on Mixtures Separated, Stationary Phases Used, and Conditions of Operation

Mixtures	Stationary phase (supported on graded Celite 535)	Column length (ft.); diameter (mm.); pressure (cm. Hg); gas velocity (ml./min); temperature (°C.)
Paraffins (765), C_5–C_7, 10 μl. total	"Dinonyl" phthalate	6/4; 74 cm. inlet; 40 outlet; 20; 65°
n-Paraffins (445), C_5–C_7	Nitrobenzene	15°
Hydrocarbons (765)	"Dinonyl" phthalate	6/4; 76 to 50; 10; room temp.
Olefins (765)	"Dinonyl" phthalate	6/4; 76 to 50; 10; room temp.
Aromatics[a] (765), C_6–C_8, 10 μl.	"Dinonyl" phthalate	6/4; 50 at inlet, 18 at outlet; 20; 80°
12 aromatic hydrocarbons (562), 60 μl.	Silicone 702	27.4 ml./mm.; 132°
Aliphatic chlorides (445)	Tritolyl ("tricresyl") phosphate	56°
Alcohols (765), C_1–C_5, 20 μl.	"Dinonyl" phthalate	6/4; 50 at inlet, 18 at outlet; 20; 80°
7 alcohols (562), C_2–C_5, 7 μl.	Silicone 702	11.8 ml./min.; 76.9°
Ethers, lower (765), 20 μl.	"Dinonyl" phthalate	6/4; 75 to 20; 25; room temp.
Oxides (765), C_2–C_5, 20 μl.	"Dinonyl" phthalate	6/4; 75 to 20; 25; room temp.
Fatty acids (441), C_1–C_5	Silicone-stearic acid 9:1 (w/w)	4/4; 46 cm.; 45 ml./min.; 100°
Fatty acids (441) C_1–C_5	Silicone-stearic acid 9:1 (w/w)	11/4; 74; 18.2; 137°
C_1–C_3 (441)	Silicone-stearic acid-orthophosphoric acid	4/4; 70; 50; 100°
C_2–C_{11} (441)	Silicone-stearic acid	4/4; 47; 18.2; 37° to octanoic acid, then 76.5 cm. Hg; 35 ml./min.
7 esters (562), 4.9 μl.	Silicone 702	11.7 ml./min.; 77°
Methyl and ethyl esters (765), C_1–C_4 acids, 75 μl.	"Dinonyl" phthalate	6/4; 74 to 17.5; 30; 57°
Carbonyl compounds (765), C_1–C_5, 50 μl.	"Dinonyl" phthalate	6/4; 74 to 13.5; 20; 78°
Amines[b] (438), C_1–C_5 normal; some iso, 2°, 3°	Mineral oil	4/4; 75; 5.7; 100° or 225 mm.; 18.7; 100°
Amines (438)	Lubrol MO (polyethylene oxide)	4/4;
Pyridine Picolines Lutidines Collidine (438)	Mineral oil	4/4; 19; 15; 137°
Mixed types (445)	Dibutyl phthalate	45 ml./min., 56°

[a] *m*- and *p*-xylenes not resolved.

[b] Diethyl, *sec* butyl, isobutyl not resolved; triethyl and *iso* amyl not resolved; *sec* butyl, diisobutyl, and cyclohexyl not resolved; all n-propyl and n-heptyl zones overlap.

Note: This table is meant only to be representative. Johns-Manville C-22 firebrick, pulverized and screened to 35–80 mesh has been reported to provide a superior support with a dimethylsulfolane stationary phase (232a, 297a), and Pelletex carbon (Godfrey Cabot Co., Boston) has been used with a squalane stationary phase (258a).

the kieselguhr mixture: with the column approximately vertical it is pressed against the rotating shaft of an electric motor (7000 rev./min.) the shaft having a flat machined on it. When all the kieselguhr has run into the column, the tube is pressed against the motor shaft until the kieselguhr packs down tightly, and this is assisted by a (polyethylene) plunger attached to a length of steel wire. This process is repeated until 4 ft. of the column is packed. . ." A wad of glass wool is then pressed down on the packing. The use of a plunger to pack down the contents of the column may in many cases not be necessary.

The packed column is adjusted in a jacket which can be heated by the condensing vapor of a suitable liquid. In the simple apparatus of James and Martin (Fig. V-1), the heating liquid must not attack the rubber stoppers used. Suitable liquids are methanol, b.p. 65.4°C.; water, 100°C.; Cellosolve, 137°C.; and ethylene glycol, 200°C. In Ray's apparatus (Fig. V-2) any safe liquid may be used. With the higher boiling liquids an air condenser is used.

James and Martin have described simple devices for controlling the **input pressure** and reading the velocity of the gas at the exit of the column. These are shown and described in Fig. V-3,*a*,*b*. Manostats to control positive or negative pressures can be obtained from laboratory supply houses, and the gas flow can be metered with a conventional flow meter or Rotameter.

A final and most important part of the apparatus is that used for the **detection of zones** of separated material at the exit of the tube. Acids (441) or bases (444) can be **titrated,** and James and Martin have described quite a simple recording automatic burette for routine use. This plots the increments of alkali used in a titration as the zones emerge from the column. Müller (667) has described an automatic titrator that records directly the first derivatives of these increments. James, Martin, and Smith (444) have illustrated simple manual devices for microtitrations. Micro and ultra microburettes can be obtained from laboratory supply houses.

Whatever method of titration is used, the gas from the column is bubbled through the indicator fluid in a closed vessel into which the microburette also dips, and thence, stripped of its acid or base, passes to the flowmeter. If manual titration is used it is convenient to add fixed increments of standard acid or base to the titration vessel and to time the change in color as the reagent is just used up by the emerging base or acid of a zone. A convenient indicator for lower aliphatic acids or amines is a saturated aqueous solution of methyl red, and the titration agents are, conveniently, $0.04N$ solutions of acid or base.

More general methods of analysis, applicable to neutral materials, have

been described. Thermal conductivity cells are often used for recognizing zones as they issue from a column (667). They are sensitive and responsive *when properly set up* (232a,597b,707a), but have some limitations in that, for example, the hot wire may decompose very thermolabile substances (247a). This is not a serious limitation. Griffiths *et al.* (352)

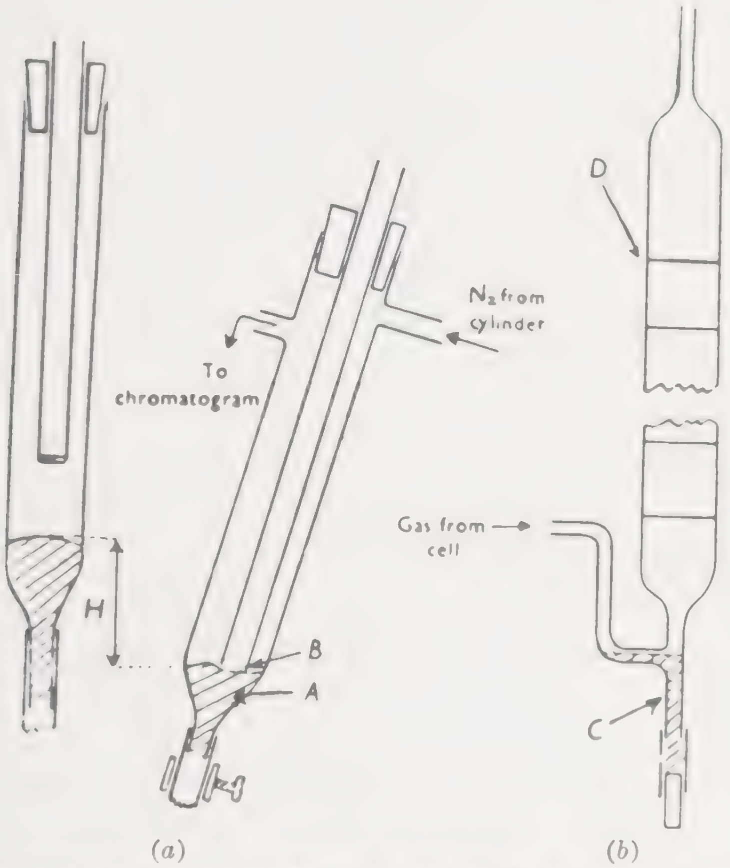


Fig. V-3. Controls for columns (*a*) Manostat. Gas from the cylinder enters, displaces mercury *A* against the adjustable head *H* until the level of the adjustable tube *B* is reached. The escape tube is closed with a piece of filter paper at *B* fastened on with sealing wax. (*b*) The exit gas from the column passes into the burette *D*, over the strong soap solution *C*. When the rubber tube is squeezed momentarily, soap solution fills the side arm, and a bubble is blown into *D*. The rate of rise of the bubble film past the calibrations is then timed. (From James and Martin (441).)

report studies on flow-impedance methods, surface-potential methods, dielectric constant methods, and others. Some of these show considerable promise of sensitivity, selectivity, and stability. A rough estimate of sensitivity given by Griffiths, James, and Phillips (352) was "1 part (molar) in 5,000 by thermal conductivity, 1 part in 1,000 by flow impedance measurements, with the simple level recorders, and 1 part in

1,000,000 by the surface-surface potential device then being studied" (353).

James and Martin (442) describe an ingenious gas density balance that is so sensitive that it can detect, for example, a concentration of 1 molecule of amyl alcohol in 50,000 of nitrogen in the gas stream (Fig. V-4). This is a most versatile kind of zone indicator, and its sensitivity and ruggedness recommend it highly.

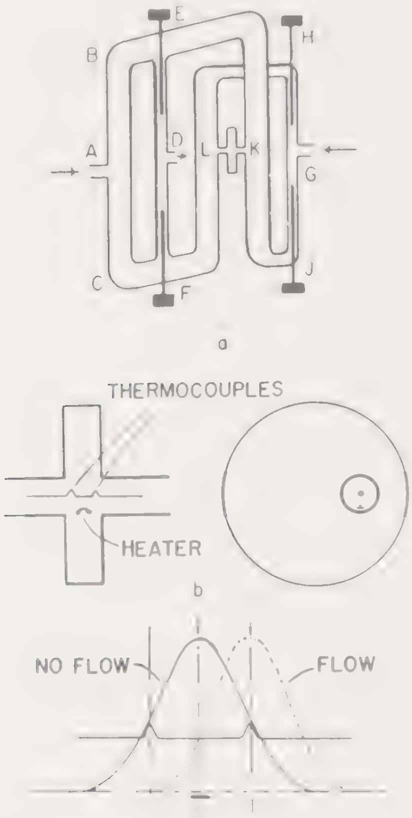


Fig. V-4. Gas density balance. (*a*) The gas from the chromatography tube and the reference stream of empty carrier gas enter at *A* and *G* respectively, exiting at *D*. *E*, *F*, *H*, and *J* are rods that can be screwed in and out, with which the flow of gas in the two arms of the bridge can be adjusted. The detector, shown in section, and end view in (*b*) lies between *L* and *K*. It contains a heater which produces a steady, symmetrical distribution of heat (*c*) about the two thermocouples when the bridge is adjusted and there is no flow between *L* and *K*—a condition which is achieved by balancing (with *E*, *F*, *H*, *J*) the streams of pure carrier gas entering *A* and *G*. However, when a zone of solute enters *A*, the density in this arm changes unsymmetrically, and the denser zone mixture causes a slight displacement of gas from *L* through *K*, "blowing" the heat away from one thermocouple toward the other. This imbalance is registered in the usual way. (Courtesy of Dr. A. J. P. Martin.)

Presumably, specialized methods of detection of zones could be used with profit in special cases: optical methods, chemical methods employing specialized indicators or other reagents, as suggested in the example above, gas-analysis cells, or biological methods for odor, or other activity. As in all chromatography, the detection of separated zones is a most crucial part of the method.

III. PROCEDURE

In carrying out an actual analysis, the column containing a suitable stationary phase is heated to the appropriate temperature (see below, and Table V-1). Sometimes it is desirable to change this temperature during the course of a run (247a,352). A few microliters of the mixture to be analyzed are deposited on the wad of glass wool at the entrance to the column, or is injected at this point, and the gas flow is started. The complete analysis requires a knowledge of the pressure of the entering and emerging gas, and the velocity of flow at some point in the setup where the pressure is known (usually at the exit), as well as the time of emergence of the peak of each zone from the beginning of the experiment. Under controlled conditions it then becomes possible to make an analysis that is highly reproducible and that gives some qualitative information and quite a high order of quantitative information.

By the use of known reference substances it is possible to identify the components of a mixture. This is possible because under controlled conditions the volume of gas that emerges up to the center or peak of the zone, the "retention volume" V_R of the center of the zone, is characteristic of a substance, and is very little affected by the other components present. Thus a column may be calibrated for known substances, and the V_R values used to suggest the nature of the components of a mixture of unknown composition. The identification of a component is further aided by adding some of the known compound to the mixture suspected to contain it, whereupon the zone at the corresponding V_R should be increased in quantity by the added amount of substance but should remain with unchanged V_R. The identification is made more certain by repeating the procedures using a stationary phase of quite different polarity. This is necessary because of the existence of constant distribution mixtures.

IV. QUANTITATIVE THEORY

James and Martin (440,441) have derived a theory for gas partition chromatography along the lines of the liquid–liquid partition chromatogram (Chapter IV). Three simplifying assumptions are made: that the

distribution coefficient of the solution is constant, i.e., a does not change over the range of concentrations in the process; that diffusion within either phase along the length of the column is negligible (this is required in the derivation of the number of plates in the column); and that the substances being separated are in so dilute a concentration that their partial pressures are negligible compared with that of the carrier gas. The following section is taken from James and Martin (441).

"The theory of gas-liquid chromatography differs from that of liquid–liquid chromatography, where a constant partition coefficient is assumed, only by virtue of the fact that the mobile phase is compressible and thus produces a gradient of gas velocity down the column. It is assumed that the pressures of the substances to be separated are negligible in relation to that of the carrying stream of gas. Let

p_1 = pressure of gas applied to chromatogram.
p_0 = pressure of gas at outlet.
p = pressure of gas at point distant x from the outlet.
V = linear velocity of gas at point x.
l = length of column.
t = time which elapses before the center of the zone emerges from the column.
F = volume of gas emerging from outlet in unit time.
$\mathbf{V_R}$ = tF = the retention volume of the centre of the zone.
$\mathbf{V_R^0}$ = limiting value of $\mathbf{V_R}$ as p_1/p_0 tends to unity.
a = the area occupied by the gas phase in any cross section of the column.
K = column constant, a function of the viscosity of the gas phase and the tightness of the column packing.

"Then

$$K\frac{dp}{dx} = \frac{Fp_0}{p} = av.$$

Integrating to give the pressure along the column:

$$K\frac{p^2}{p_0} = 2Fx + Kp_0,$$

or

$$K = \frac{2Fxp_0}{p^2 - p_0{}^2},$$

or

$$F = \frac{p_0K}{2x}\left[\left(\frac{p}{p_0}\right)^2 - 1\right]. \tag{1}$$

now

$$t = \int_0^l \frac{dx}{vR_F} = \int_0^l \frac{apdx}{R_Fp_0F}. \quad (2)$$

Here R_F has the usual chromatographic meaning, that is, it represents the rate of movement of the zone of the substance under consideration relative to the rate of flow of the moving phase. In the present case the rate of flow of the moving phase increases continuously as it advances down the column, but the R_F remains constant and the rate of movement of the zone increases correspondingly. The R_F is a constant depending only on the substance to which it refers, the temperature, the nature and amount of the stationary phase and, in so far as the support is not inert, on its amount.

"Rewriting Eqn. 2 in terms of pressures,

$$t = \int_{p_0}^{p_1} \frac{Kap^2dp}{R_FF^2p_0^2} = \frac{Ka(p_1^3 - p_0^3)}{3R_Fp_0^2F^2},$$

$$\mathbf{V_R} = Kap_0 \frac{[(p_1/p_0)^3 - 1]}{3R_FF}.$$

Hence, by (1),

$$\mathbf{V_R} = \frac{2}{3}\frac{al}{R_F}\frac{(p_1/p_0)^3 - 1}{(p_1/p_0)^2 - 1}. \quad (3)$$

"As p_1/p_0 tends to unity then $\mathbf{V_R}$ tends to $\mathbf{V_R^0} = al/R_F$. In fact, the flow rate V is measured at room temperature and should be corrected to the column temperature.

"Since the value of R_F is not dependent on p_0 it is not possible by reducing p_0 to reduce the temperature of operation of the column. Further, since the viscosity of the gas is independent of the absolute pressure, when p_0 is low, the ratio of the pressures at the two ends of the column becomes excessive if a reasonable rate of flow is to be maintained. Under normal conditions, it is convenient for p_0 to be atmospheric pressure. It might, however, be preferable to increase p_0 by a factor of approximately 10 if a very long column is to be used in order to effect a difficult separation. This is due to the fact that the absolute pressure drop from one end of the column to the other for a given retention volume will be independent of p_0; consequently it will be possible to work at a low ratio of p_1/p_0 even with a very long column.

"*Test of relationship between pressure, flow rate and retention volume:* An experimental test of the theory given above was carried out by the procedure which is described later in the paper. A 4 ft. silicone-stearic acid column was set up and a series of separations of acetic, propionic, *n*-

and *iso*-butyric acids carried out at four different pressures. The flow rates were measured in each case and from the graphs the retention volume of each acid band was measured.

"The theoretical ratio of flow rates is given by (1):

$$\frac{F_1}{F_2} = \frac{(p_1/p_0)^2 - 1}{(p_2/p_0)^2 - 1}.$$

"In Table V-2 the theoretical ratios of the flow rates are compared with those experimentally determined; agreement is within expected error.

"From Eqn. 3 it can be seen that as the ratio of the pressures at the two ends of the column tends to unity the factor

$$\frac{(p_1/p_0)^3 - 1}{(p_1/p_0)^2 - 1}$$

TABLE V-2

Comparison of Calculated with Experimentally Determined Flow Rates in a Gas-Liquid Partition Chromatogram Operated at Different Pressure Ratios (441)

(The rates in runs a–d (Table V-3) are compared. For basis of calculation see text)

	Flow Rate Ratios Calc.	Found
a/b	1.44	1.46
a/c	1.92	1.96
a/d	3.36	3.21

tends to 3/2. At this limiting pressure ratio the retention volumes of the bands tend to a limit V_R^0. In Fig. V-5 the values of p_1/p_0 are plotted against the value of the ratio V_R/V_R^0; by means of this graph the experimental value of V_R can be corrected to V_R^0. In Table V-3 these values of V_R^0 are shown for four experiments carried out at different values of p_1/p_0. It can be seen that the figures agree within 5%, a fairly satisfactory confirmation of the theory, since the assumption is made that the column is uniformly packed.

"*Derivation of column efficiency.* Two methods of calculating column efficiency from the experimental curves are given in order to allow comparisons with one another and with data from liquid–liquid columns.

"*Method 1*

"Let there be r plates in the column.

"Assume that all the material (M equivalents) is put on in the first plate. Then the quantity occurring in the last plate at the maximum concentration is $M/\sqrt{(2\pi r)}$ equivalents.

"Let q be the fraction of the original quantity occurring in any plate and let the time taken for the centre of the zone to reach the last plate be

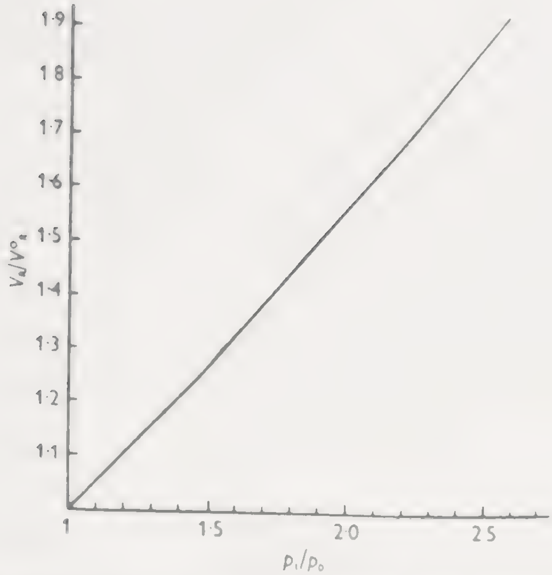


Fig. V-5. The relationship between the pressure ratio across the column and the ratio between the observed retention volume and the retention volume at zero pressure difference across the column. (From James and Martin (441).)

TABLE V-3

Correction of Retention Volumes to Their Limiting Values (V_R^0) for Runs at Different Pressure Ratios (441)

Run	Pressure of N_2 applied to column (cm. Hg)	p_1 (N_2 pressure plus atmospheric pressure, p_0) (cm. Hg)	p_1/p_0	V_R^0 (ml.) Acetic acid	Propionic acid	Isobutyric acid	n-Butyric acid
a	76.0	150.91	2.014	203	466	758	952
b	57.1	132.0	1.762	193	457	749	932
c	45.7	120.61	1.647	194	455	740	922
d	28.9	103.81	1.385	193	464	763	954

t min.; then the time taken for contents of last plate to escape $= t/r$ min.

"Then $M_{q\max.} = M/\sqrt{(2\pi r)}$ equivalents and $M_{q\max} r/t =$ max. rate of escape $=$ max. slope of the experimental curve $= S$ equiv./min.

"Then

$$S = Mr/t\sqrt{(2\pi r)} \text{ equiv./min.}$$

"Therefore

$$r = 2\pi S^2 t^2/M^2.$$

"Thus by measuring the maximum slope of the effluent concentration curve, the time to arrive at the maximum slope and the total amount of the

material in the zone, the plate number of the column and hence the height equivalent to a theoretical plate (H.E.T.P.) can be obtained. H.E.T.P. is defined for the chromatogram by Martin and Synge.

"*Method 2.*

"The area between ordinates one standard deviation on either side of the peak of an error curve is 68.3% of the total. From the theory given by Martin and Synge the shape of the chromatographic zone should approximate to an error curve and 68.3% of the material should be included between plates $\pm\sqrt{r}$, where r is the total plate number.

"The ratio of time taken for the centre of the zone to emerge (t) to the time taken for the middle 68.3% of the zone (τ) is equal to $r/2\sqrt{r}$.

"Hence

$$r = 4t^2/\tau^2.$$

"A comparison of figures obtained by the two methods for the same column gives fair agreement (Table V-4), the agreement increasing with the plate number."

Experimental tests of this theory agreed well with it (440,441). For example, with a certain column, the acids acetic, propionic, normal, and

TABLE V-4
Comparison of Column Efficiencies Obtained by Two Independent Methods (441)
(For methods 1 and 2 see text)

Flow rate (ml./min.)	Plate number calculated by Method 1	Method 2
35	550	400
18.2	718	600
10.3	765	730

isobutyric were separated at four different pressures. The calculated V_R values agreed well with the experimentally determined, and also the V_R^0 values calculated from experiment for each acid were essentially constant over the range of pressures, agreement within 5% being obtained.

Remarkable column efficiencies in terms of numbers of theoretical plates were calculated for some of the columns, using the methods described above. These efficiencies depended on rate of flow of gases, uniformity of packing, dispersion of the stationary phase, temperature, nature of the stationary phase. Thus by the two methods of calculation given above a certain column showed 550 and 400 plates at a flow rate of 35 ml. gas per minute. In another run, when the flow rate was decreased to 10.3 ml./min. the plate numbers were calculated to be 765 and 730. With a number of different 4-ft. columns plate numbers from 700 to 1200 were found, and an 11-ft. column showed 2000. One column run at 65°C. showed 365 plates

When run at 100°C., the efficiency had increased to 720 plates. James and Martin (441) point out that the higher the viscosity of the stationary phase the less efficient the column will be because the rate of diffusion of solute is less the more viscous the liquid.

The effect of increase in operating **temperature** of a column will be to decrease the ratio of retention volumes of homologs, for, as James and Martin say (441) "in a homologous series the higher members will have larger latent heats of vaporization, their change of vapor pressure with temperature will be larger than with the lower members, so that the factor of separation will decrease as the temperature increases." For example, the ratio $\mathbf{V_R}$ of propionic acid/$\mathbf{V_R}$ of acetic acid decreased from 2.9 at 65°C. to 2.1 at 137°C. That this effect also applies to isomers is shown by the decrease in the $\mathbf{V_R}$ *n*-butyric/$\mathbf{V_R}$ iso-butyric from 1.34 at 65°C. to 1.20 at 137°C.

van Deemter and co-workers (957a) and Littlewood (561a) have developed theories for gas partition chromatography which, because of the mechanism on which they are based, permit an additional insight into the effects of changing operating variables. The theories are similar, and that of van Deemter, Zuiderweg, and Klinkenberg (957b) has been outlined, with some experimental support, by Keulemans and Kwantes (469a). For this reason the formulation of these workers is utilized, and their and Littlewood's discussions are related to it.

The model used by these workers describes a chromatographic analysis in terms of the rate of broadening of the zones, initially assumed to be very sharply defined, and very narrow, as they move along the column. This broadening is related to H.E.T.P., and hence to the efficiency of the column, as shown below. In this model it is assumed that the column is uniformly packed, so that the ratio of the gas to liquid phases is essentially constant along the column. Then, the broadening of the zone is produced primarily by three phenomena. (*a*) One is an "eddy diffusion" which is a concomitant of the presence of the packing. Its magnitude depends on the size of the particles, their shape, and how they are packed. In effect, since there are many paths of unequal lengths and widths and in irregular directions between the particles of packing, the molecules traveling in the gas phase are spread out in terms of their residence time in the column. This causes a spreading of the zone with distance traveled. A similar effect was noted in adsorption chromatography (Chap. VIII, IV, 2,5,6). (*b*) Another is the molecular diffusion in the gas phase (that in the liquid is assumed to be negligible). This tends to broaden the zone with time. (*c*) The third phenomenon is caused by lack of instantaneous equilibrium between mobile and stationary phases. Because of it some molecules may fail to go into the stationary phase and so travel faster

than equilibrium would predict, while at the same time some molecules are slow in moving from stationary to mobile phase and thus lag behind the main zone. This tends to broaden the zone and is termed "resistance to mass transfer." The second zone-broadening effect is due to "longitudinal diffusion" and the third to "lateral diffusion" in Littlewood's terms.

Without derivation, the relation of van Deemter, Zuiderweg, and Klinkenberg is given by Keulmans and Kwantes as:

$$\underset{(a)}{H = 2\lambda d_p} + \underset{(b)}{\gamma D_{\text{gas}}/u} + \underset{(c)}{(8/\pi^2)[k'/(1 + k')^2](d_f^2/D_{\text{liq.}})(u)} \quad (4)$$

where the letters (a), (b), (c) refer to the contributions of the diffusion effects listed above. The symbols of these authors are used since the parameters are largely different from those used by James and Martin in the previous equations. H is the height equivalent to a theoretical plate (H.E.T.P.). (*a*) λ is a quantity characteristic of the packing, and d_p the average particle diameter. (*b*) γ is "a correction factor accounting for the tortuosity of the gas channels," D_{gas} is diffusivity of the zone substance in the gas phase, and u is the "linear gas velocity in the packed column." (*c*) k' is defined as $K(F_{\text{liq.}}/F_{\text{gas}})$, where K is the partition coefficient of the zone material (the isotherm of which is assumed to be linear) expressed as the ratio of moles per unit volume of stationary phase to moles per unit volume of mobile phase, $F_{\text{liq.}}$ and F_{gas} being the fraction of cross section of the corresponding phases. d_f is an "effective thickness" of the film of stationary phase, and is directly proportional to the amount of stationary phase. $D_{\text{liq.}}$ is the diffusivity of the zone substances in the liquid phase. It may be expected that an expression of this kind may be modified later as knowledge accumulates about the factors that affect efficiency, but this does not seriously impair its present use in examining the effects of changes in operating conditions, since the utility of the expression has been shown by Keulemans and Kwantes. Ten operating conditions subject to control are enumerated by Littlewood.

(*1*) Littlewood points out that it has been shown many times that "doubling the number of plates" by doubling the length of a column does not double separating efficiency but rather, multiplies it by $\sqrt{2}$, tripling the length multiplies it by $\sqrt{3}$. Here efficiency is defined in terms of the spread of the zones.

It is to be observed that a small value of H (in equation 4) is desirable, or, stated another way, an increased number of theoretical plates, since, apart from the length variable, this is a direct measure of the efficiency of a given column. Then the effects of various conditions on H are obtained from equation 4 as follows.

(2) As Keulemans and Kwantes point out, equation 4 may be written in the form:

$$H = A + B/u + Cu \qquad (5)$$

This is the equation of a hyperbola with a minimum at $u = \sqrt{B/C}$. The plot of H vs. u shows a minimum at this point, which represents the most efficient **velocity of mobile phase.** At slower rates of flow, decreased efficiency is largely determined by the gas-diffusion effect (b); at rates of flow higher than this maximum efficiency value, lowered efficiency is largely caused by resistance to mass transfer (c). In most cases, however, a column is likely to be operated at rates somewhat higher than the maximum efficiency value, in order to get the job done, and this case will be considered in what follows. (See Keulemans and Kwantes for actual data on H vs. u.)

(3) A discussion of **retention volume** is in practice a discussion of the partition coefficient K. At a high flow rate equation 4 takes the form such that for increased efficiency k' is large compared to 1. Then the term $k'/(1 + k')^2$ reduces to $1/k'$. Then, with high flow rate, the higher the retention volume, the greater is the efficiency. The interpretation of the effect is probably not simple because $D_{liq.}$ is quite likely to decrease with the increase in k'. Both k' and $D_{liq.}$ are contingent on molecular properties. Practically speaking, however, the effect must be expected to be as stated.

(4) K increases with decrease in **temperature** because of the heat of solution effect of solute in the stationary phase. But a decrease in temperature will increase the viscosity of the stationary phase, as James and Martin also pointed out, and hence lower D_{liq}. Which effect predominates in practice depends on the nature of the stationary phase, and was discussed in connection with James and Martin's theory, above. When the column is overloaded, rise in temperature tends to increase efficiency.

(5) According to the theory of equation 4, the nature of the carrier gas affects only the molecular diffusion term (b). In cases where this term decreases in importance, namely at high flow rates, the nature of the carrier gas becomes less important, though a less viscous gas is to be preferred. Under these conditions a low value of the resistance to mass transfer (c) in equation 4 improves efficiency. At low flow rates, where the "molecular diffusion" term increases in relative importance, the nature of the gas may exert a profound effect. In general, under such conditions, low molecular weight gases lead to poor efficiency, and nitrogen (say) is better than helium or hydrogen, *other things being equal.* Practically, the efficiency of the column usually being great enough for its further improvement to take a secondary place in consideration, the choice of carrier gas is governed largely by availability and the require-

ments of the detection system. For example, when zones are to be detected by thermal conductivity differences the choice of helium as carrier gas may be overriding.

(*6*) Apart from selectivity effects, which are discussed from a structural point of view in the following section (V), it can be said here that it is preferable from the point of efficiency that the **stationary phase** be a good solvent for the zone material (this increases K). Put in another way, the vapor pressure of the substance above the stationary phase should be less than ideal. It is of some importance that the molecular weight of the stationary phase should be as low as practical in order that the mole fraction of zone material be as low as possible, and also that the viscosity of the stationary phase be low.

(*7*) Since k' is to be large (see (*3*)) $F_{\text{liq.}}$ should also be large; hence it may appear that the greater the **mass of stationary phase per unit length of column** the better. However, counter to this is the d_f term, which, occurring as a square, will very likely more than overbalance the other term, so that in fact increase in $F_{liq.}$ is useful only for small values of K, and these in themselves are not practical since small K means that the sample used must be small. Moreover, adsorptive effects (Section V) may then supervene.

(*8*) From the argument in (*3*), at higher flow rates efficiency decreases with increase in **free volume** (F_{gas}). At the same time, the larger F_{gas} the more important the term (*b*) in equation 4 is likely to become.

(*9*) Decrease in **particle size** of the support, keeping the mass of the stationary phase per unit length of column constant, would decrease film thickness and increase surface area of contact with the mobile phase, thus tending to improve mass transfer and with it efficiency if the support is nonporous. With quite porous particles, these quantities are largely determined by the internal structure, and so would be less affected by decrease in particle size. However, because of the difficulty of obtaining regular packing, an intermediate mesh size is preferable. According to Keulemans and Kwantes a range of particle size between 30 and 80 mesh A.S.T.M. contributes to the H.E.T.P. about 0.1 cm. independent of the operating conditions.

(*10*) **Presure drop along the column** has several effects. The effect on retention volume was discussed by James and Martin, quoted above. There is no theoretical treatment of the effect on zone-spreading but probably, since the zone occupies a relatively small region of the column, across which the pressure drop will be small, the effect will be comparatively small. The effect of the velocity gradient produced by the pressure drop may be that the flow rate for maximum efficiency is straddled. In general, much of the column would be operating not too

far from this favorable velocity. According to Keulemans and Kwantes the pressure drop along the column has to be rather large before it becomes a serious factor.

Several other factors, not specifically dealt with in equation 4 may be mentioned here. It has been found that *column diameter* has in general not a very great effect on efficiency. There is in equation 4 nothing about the effect of the quantity of solute. It is, however, observed that increase in quantity of the sample tends to decrease efficiency, partly because it becomes more difficult to obtain a sharp initial zone ("charging all the material into the first plate"); partly because a partial fractionation may occur during introduction of the sample. This subject has been quantitatively described by Glueckauf (327a) and by van Deemter, Zuiderweg, and Klinkenberg (957b).

V. FACTORS THAT AFFECT V_R

(See Table V-5). The factors that affect V_R (or **R**) in gas chromatography may be discussed at various levels: in terms of factors that clearly affect the entire system, as the temperature, pressure, concentration; and in terms of interactions that may as a convenient approxima-

TABLE V-5
Factors That Affect V_R

Properties of

- The system
  - Concentration
  - Pressure
  - Temperature
- The stationary phase (seat of most important interactions)
  - Interactions between
    - Solute and supported phase (effects on α)
      - Dispersion interactions (effects of molecular weight and shape)
      - Hydrogen bonding
        - Stationary phase as an acceptor
        - Stationary phase as a donor
      - Chemical reaction
    - Molecules of supported phase
      - Associations, competitive with interaction with solute
    - Molecules of solute
      - Associations, depolarizing effects
    - Support and solute
      - Adsorptions
- The mobile phase
  - Associations of solutes (?)

tion be localized to the mobile phases, the stationary phases, and the supporting phase. However, these latter are contingent. A factor that affects behavior of V_R via the mobile phase usually influences also the stationary phase. It must be stressed that though we try to isolate the more important variables we are *always dealing with a system in which all parts are mutually dependent.* (See Tables VII-5, 6, 7, 10.)

Approach to ideal behavior in a system is favored by lowered concentration, or pressure, and often by elevated temperature. Thus most distribution isotherms (Chapter IV) approach linearity at sufficiently low concentrations. In gas chromatography dilute systems are the rule, as well as elevated temperatures. In addition, because the mobile phase is gaseous, and the stationary phase is heated, rapid diffusion of solution between and in the phases is favored, with the chance for rapid approach to equilibrium. All these factors tend to good agreement between practice and theory (as is indeed found).

There seem not to be any very important factors that affect V_R specifically through the mobile phase. In the case of fatty acids (as an example) very likely the acid molecules are dimerized and trimerized to some extent in the carrier gas. This would increase their effective molecular weights in this phase. But apparently the gas phase acts almost exclusively as an inert carrier, and is of minor importance in influencing V_R. This is intentional. Of course, a *reacting* gas could be used which, however, would alter the picture. Azeotrope formation would also alter the picture (see Section VIII).

Many interactions that affect V_R occur in the stationary liquid phase, but only those between solute and solvent, a convenient term for the supported phase, affect the separation directly, so to speak. The rest influence the separation by competing with these.

Interactions between solute and solvent affect V_R through α. It will be noted that V_R is related to α in this way: a substance that tends more to remain in the stationary phase than in the mobile will have a *higher* V_R than one that tends to stay more in the mobile phase, because a zone of the latter will issue from the chromatographic tube sooner, with a lesser volume of carrier gas preceding it than the former will. Since the mobile phase has little influence on V_R through interaction with the solutes, the importance of the stationary phase becomes very apparent.

Factors that tend to keep molecules in the stationary phase will increase α, decrease R *and* R_F, *and increase* V_R.

If the stationary phase is neither a donor nor acceptor in hydrogen bonding, and nonpolar, for example, a *paraffin* hydrocarbon, such as mineral oil, and the solutes are hydrocarbons, then presumably the interactions between solutes and stationary phase will be largely of

the dispersion type. This is reflected in the separations that occur according to molecular weight and shape (Fig. V-6) and which are related also for the same reasons to relative volatility. A similar relation is found when the stationary phase is a paraffin and the solutes are amines (Fig. V-7). When an attempt was made to separate lower fatty acids using the paraffin-like silicone as stationary phase, distorted zones were obtained (Fig. V-8). James and Martin (441) corrected this by adding 10% of

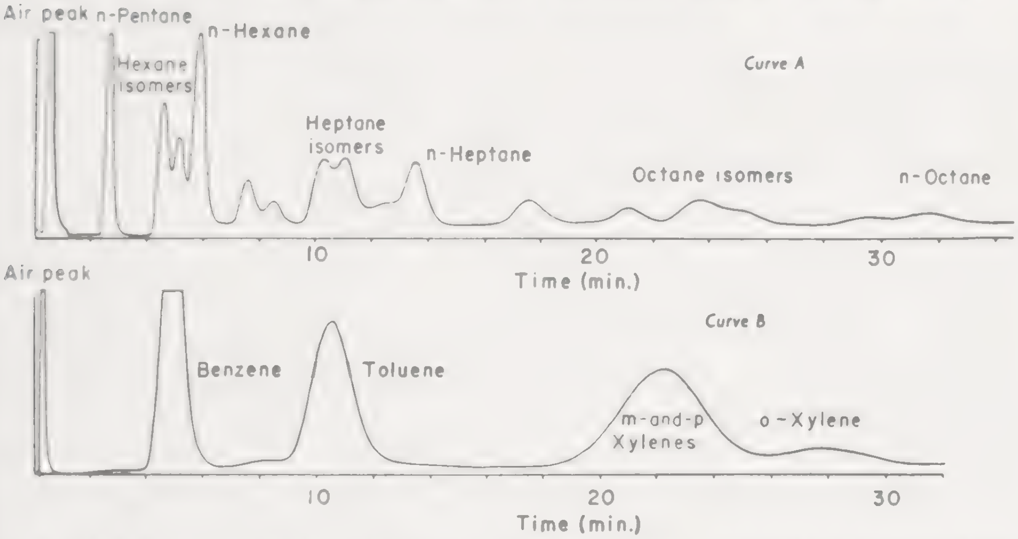


Fig. V-6. Separation of hydrocarbons. (*A*) The separation of aliphatic hydrocarbons from petroleum ethers of boiling range 40° to 120°C. Column length, 4 ft.; stationary phase, liquid paraffin; temperature, 78.6°C.; rate of flow of nitrogen, 28 ml./min.; pressure of nitrogen, 36 cm. Hg. (*B*) The separation of aromatic hydrocarbons. Column length, 4 ft.; stationary phase, benzyldiphenyl; temperature, 100°C.; rate of flow of nitrogen, 42 ml./min.; pressure of nitrogen, 42.6 cm. Hg. (After James and Martin (442).)

stearic acid (which is non-volatile at the temperatures used) to the stationary phase. They explain that without the stearic acid the zones produced by the higher acids (curve *A* in the figure) had extended front and sharp rear boundaries because regions of low acid concentration, where the ratio of monomer to dimer in the stationary phase is relatively high, tend to move along the column more rapidly than regions of higher acid concentration, where the ratio of monomer to dimer is lower, that is, low concentration regions move faster and tend to become lower in concentration, thus giving extended fronts to the zones. The reason for this behavior is that the partial pressure of the acid in the mobile phase is nearly proportional to the concentration of the monomer in the stationary phase. By swamping the solute with the large amount of stearic acid, itself capable of associating with the monomer the concentration of

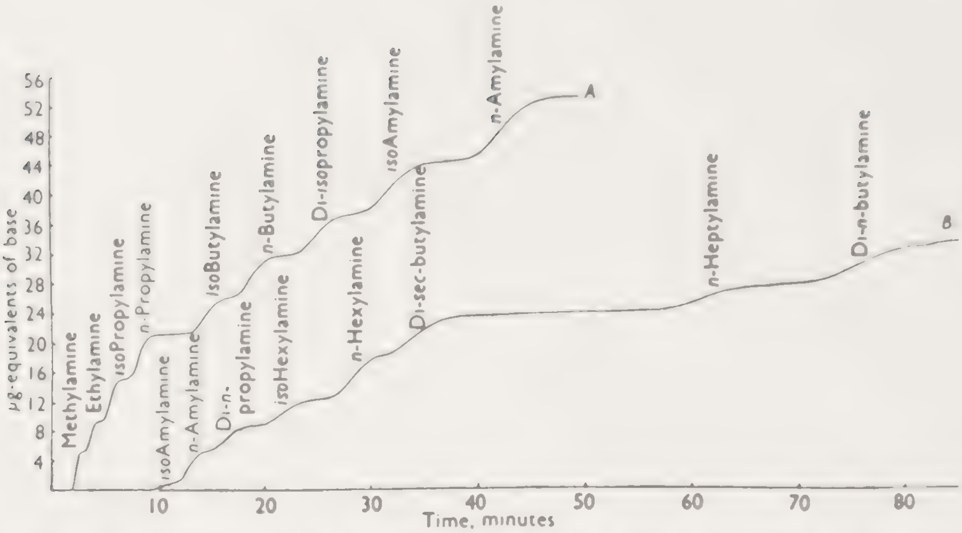


Fig. V-7. The separation of amines. (*A*) Rate of flow of nitrogen 5.7 ml./min.; pressure of nitrogen 7.5 cm. Hg. Amines in order of appearance: methylamine, ethylamine, isopropylamine, *n*-propylamine, isobutylamine, *n*-butylamine, diisopropylamine, isoamylamine (overlapping with triethylamine), *n*-amylamine. (*B*) Rate of flow of nitrogen, 18.7 ml./min.; pressure of nitrogen, 22.5 cm. Hg. Amines in order of appearance: isoamylamine, *n*-amylamine, *di-n*-propylamine, isohexylamine, *n*-hexylamine, di-sec.-butylamine (overlapping with diisobutylamine and cyclohexylamine), *n*-heptylamine (overlapping with tri-*n*-propylamine), di-*n*-butylamine. (After James and Martin (442).)

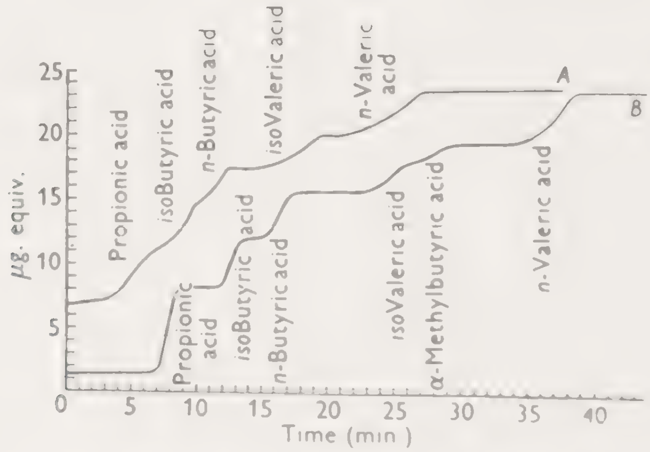


Fig. V-8. Behavior of fatty acids. The separation of propionic, isobutyric, *n*-butyric, commercial isovaleric, and *n*-valeric acids. (*A*) Column length 4 ft., stationary phase DC 550 silicone; nitrogen pressure, 30 cm. Hg., flow rate 23 ml./min.; column temperature 100°C. Incomplete resolution of all the acids. (*B*) Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC 550 silicone; nitrogen pressure, 40 cm. Hg., flow rate 45 ml./min.; column temperature 100°C. The isovaleric fraction has been almost completely resolved into two components. (After James and Martin (441).)

the latter and therefore the value of a was made nearly independent of concentration of acid, and thus the shapes of these zones approached the ideal sigmoid form and sharp separations were obtained (curve B).

If the stationary phase is an *acceptor in hydrogen bonding*, for example, an *ether* such as a polyethylene oxide ("Lubrol MO" was used), then solutes capable of forming hydrogen bonds will be retarded with respect to their hydrogen-bonding ability. This is shown in Fig. V-9, where $\mathbf{V_R}$'s of pri-

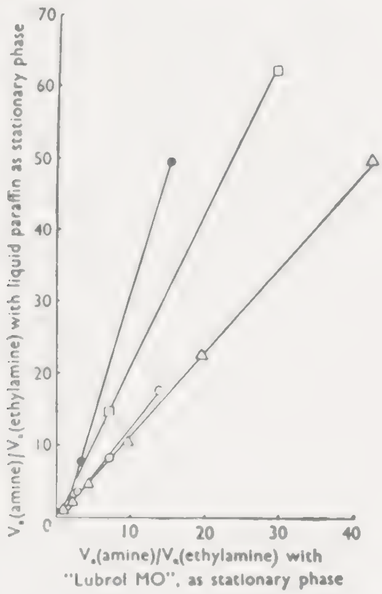


Fig. V-9. Relationship retention volume of amine/retention volume of ethylamine, for columns having stationary liquid phases of (a) liquid paraffin, and (b) a polyethylene oxide "Lubrol MO." The figure shows the relative speeding-up of secondary and tertiary amines on changing from a non-hydrogen-bonding stationary phase to one allowing hydrogen bonding. Δ: primary straight-chain amines; ○: primary amines with an *iso* configuration; □: secondary straight-chain amines; ●: tertiary straight-chain amines. (After James and Martin (442).)

mary, secondary, and tertiary amines relative to ethylamine are compared for a paraffin and a polyether stationary phase. As James (438) pointed out, the possibility of hydrogen bonding of primary and secondary amines with the ether must be considered as well as the inability to bond in the case of the tertiary amine. Thus relative to their behavior to the paraffin stationary phase, which takes account of weight and shape factors (and relative also in all cases to ethylamine), the primary amines are retarded more by the polyether stationary phase than the secondary, and the secondary than the tertiary.

If the stationary phase is a *hydrogen donor* in hydrogen bonding it would have a retarding effect on ethers, amines, esters, ketones, amides, and so on. For example, whereas when the stationary phase is a liquid paraffin methylamine, then dimethyl, then close behind trimethyl amine emerge in that order, they appear in the reverse order when the stationary phase is glycerol, or Silicone DC 550 containing 10% hendecanol (444). Of course, quite complicated effects, probably including all the van der Waals interactions as well as hydrogen bonding, are possible in a system like this, and too simple an explanation may be unwise. After all, the order of basicity of these amines is $2° > 1° > 3°$, while the boiling points fall in the order of $1° < 3° < 2°$; and it is known that steric factors play important roles in influencing the properties of these and related substances. Besides, the ever-present competitions due to association of solute, and of solvent, may play a role. At this stage the chromatographer has to make the most informed guesses possible, and not allow himself to be overwhelmed by the truly formidable difficulties in the way of *possibilities* that lurk in his data.

If the stationary phase *reacts chemically* with the solute, retardation may be very great: for example, if an acid were to react with a basic solute, or esterify an alcohol (reversibly, and even rapidly at the temperature of the system); or if aldolization or other chemical reactions were to occur.

Interactions *between molecules of the stationary phase* which lead to association would tend to squeeze out solute molecules (in the absence of solute–solvent interactions) and so decrease their retardation. This is an interaction suggested by James, Martin, and Smith (444) to explain the order of emergence of amines on a glycerol stationary phase column. The order is trimethyl, then dimethyl, then methyl, that is, the reverse of the molecular weight. They suggest that "the bonding energy between the glycerol molecules is greater than that existing between the glycerol and amine molecules, the effect being analogous to a high internal pressure forcing out the molecules of higher mol. wt." Evidence for this effect from a less complicated system would be desirable. In any event, stationary phases that are strongly associated are also likely to be capable of interacting with polar solutes. Upon this rests the possibility for fruitful choice of stationary phases.

If the solute molecules are polar and associate, this may increase their solubility in nonpolar stationary phases. This is because the association, as, for example, the dimerization of aliphatic acids in nonpolar solvents, or in the vapor phase by chelation, *depolarizes* the substance. The effect of this kind of behavior was illustrated in the case of the separation of fatty acids using a silicone stationary phase without and with stearic acid present (Fig. V-8).

The *supporting material* may influence V_R. It must be wetted by the stationary phase, and this implies some interaction. Preferably, the stationary phase, or some swamping component in the stationary phase, must be adsorbed to the surface of the supporting material more strongly than any component of the mixture being separated. When this condition is not fulfilled, the zones of substance tend to tail out: to be quite extended in the rear. For example, James and Martin (441) found that on a silicone–stearic acid column the zone of formic acid showed tailing which extended into the acetic acid zone, and made complete resolution of the two acids impossible. Upon incorporating orthophosphoric acid in the stationary phase (or on washing the diatomaceous earth support with dilute aqueous orthophosphoric acid before drying it and mixing with the stationary phase) the zone of formic acid became sigmoid and separate from that of acetic acid. These authors suggest that the tailing was due to adsorption of the formic acid to the diatomaceous earth. Adsorption is normally relatively stronger at lower concentrations, and for this reason adsorption zones tend to have sharp fronts and extended rear boundaries (tailing). The situation is fairly complicated, however, for the addition of the phosphoric acid to the stationary phase increases V_R of all the acids. Martin and James suggest that this indicates greater association in the stationary phase.

A similar effect was noticed by James, Martin, and Smith (444). In the separation of methyl-, dimethyl-, and trimethylamines in a column with a stationary phase of a hendecanol–liquid paraffin mixture supported on diatomaceous earth, the support adsorbed the amines, and each zone showed tailing. The tailing was suppressed to some extent by pretreating the diatomaceous earth with sodium hydroxide in methanol, but some tailing still remained.

One objective in preparing a good column is to eliminate entirely any interaction of the surface of the support with the solutes to be separated.

VI. A FEW RELATIONS BETWEEN V_R AND MOLECULAR STRUCTURE

A few of these may be summarized. In a series of **homologs** the retention volume increases in a regular way with the number of CH_2 groups above the first few. With *n*-aliphatic acids, except for the first two, on the silicone–stearic acid column, each additional CH_2 multiplies the retention volume by approximately 1.9 (441). This same factor applied to the *iso*-acids that were examined (Fig. V-10). With the primary straight-chain amines above ethylamine the factor for each CH_2 group was 2.1 to 2.2, and the same slope of curve was found for the primary *iso*alkyl amines.

(The isomers are separated by a factor of 1.3 to 1.4.) The factor for secondary straight-chain amines was slightly less, and that for tertiary amines, still less. Similar relationships of homology were reported by Ray (765) (see Fig. V-11).

James and Martin (442) compared **corresponding compounds** by studying the interaction of a compound C_5H_{11}-X with an aromatic stationary phase (as compared with a paraffin stationary phase) and found the

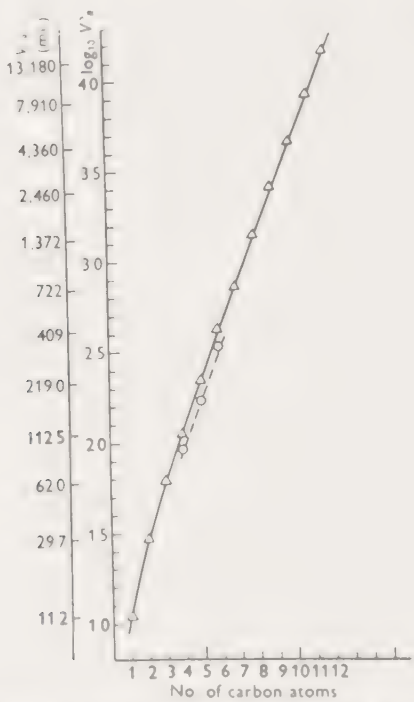


Fig. V-10. Relation of homology. Relationships between retention volume at zero pressure difference, V_R^0, and the number of carbon atoms in the lower fatty acids. Data are for a 4-ft. silicone–stearic acid column at 137° C. Δ: *n*-acids; ○: *iso*-acids. (After James and Martin (441).)

compounds to be ordered in terms of their interactions: X = H < Cl < Br < I < NH_2 < NO_2 < OH < $COCH_3$ < CN (Fig. V-12). This order does not seem to be produced by one type only of interaction. When a polyethylene oxide column was compared with a paraffin column, the order found was H < OCH_3 < Br,Cl < I < NO_2 < $COCH_3$ < NH_2 < CN < OH. The authors call attention to the probably greater role of hydrogen bonding in the latter than in the former series. They also point out that gas partition columns provide a powerful tool for studying relative interactions.

VII. CHOICE OF PHASES

The following considerations can help guide the choice of phases in gas partition chromatography.

The carrier gas is usually inert, a gas such as nitrogen being suitable. However, a gas may be used that can later be removed by reaction at the end of the column.

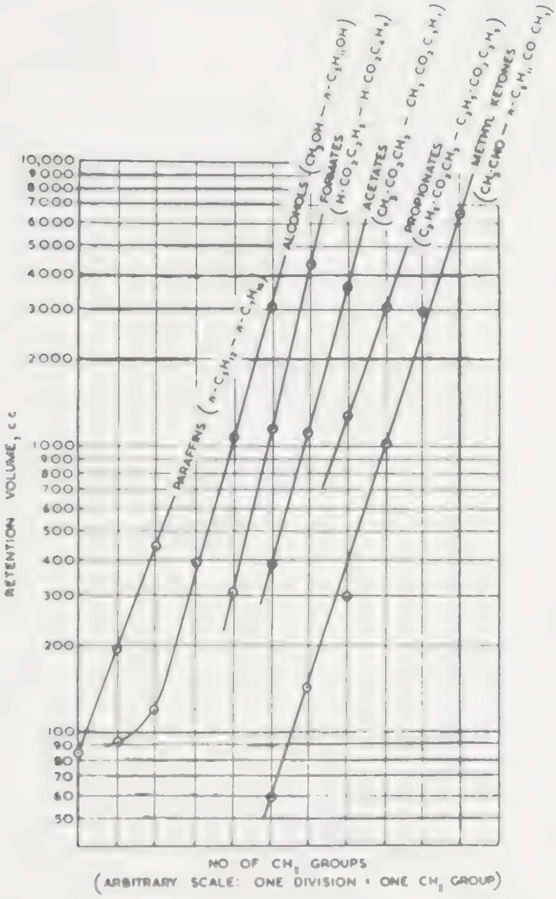


Fig. V-11. Relation between retention volume and chain length. P, n-paraffins C_5 to C_7; Al, alcohols, C_1 to C_5; F, formates $HCO_2C_2H_5$ to $HCO_2C_4H_9$; Ac, acetates $CH_3CO_2CH_3$ to $CH_3CO_2C_3H_7$; Pr, propionates $C_2H_5CO_2CH_3$ to $C_2H_5CO_2C_3H_7$; MK, methyl ketones, CH_3CHO to CH_3CO-nC_5H_{11}. (After Ray (765).)

The stationary phase should be essentially nonvolatile, but if it shows a slight volatility this can be corrected for in the analytical system by, for example, using a column packed with the same materials and using the same carrier gas but without solute, as a control.

If a stationary phase *chemically "like"* the mixture to be separated is used, for example, mineral oil to separate paraffins (see Fig. V-6), the separation will be largely on the basis of molecular weight and shape, as these are reflected in relative volatility.

If the stationary phase is *chemically "unlike"* the components of the mixture, in terms of functional groups, there will be superimposed on molecular weight and shape factors other polar interactions. For example, with a polyether stationary phase hydrogen donors will usually be re-

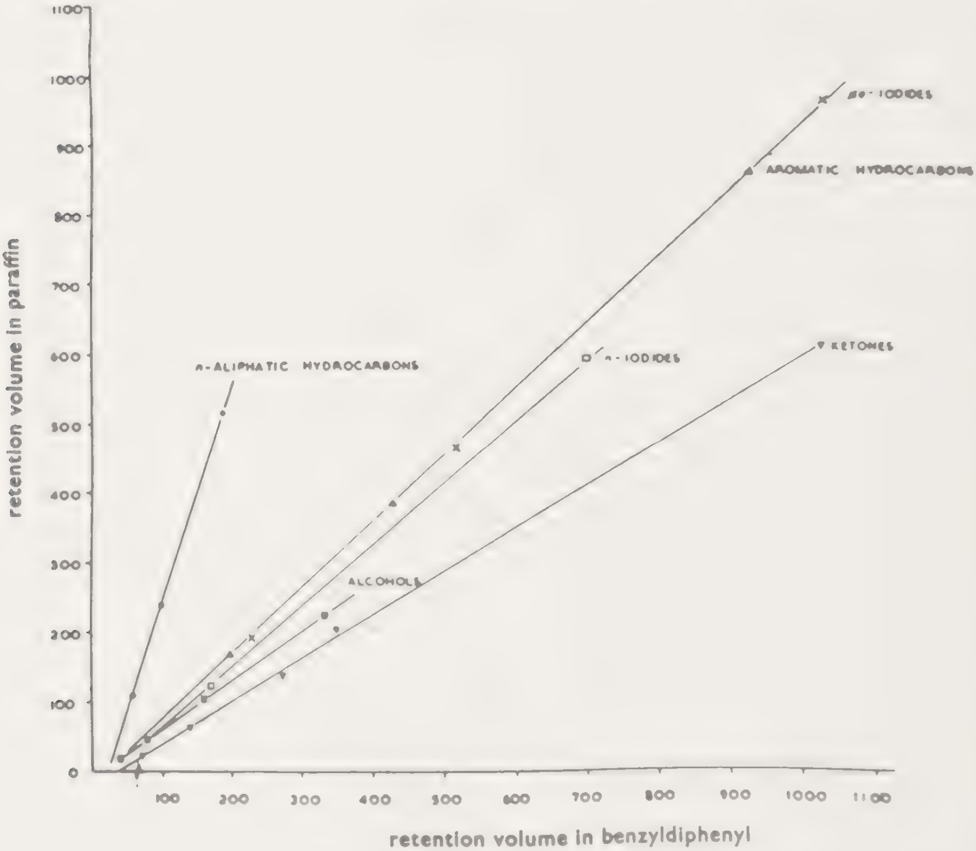


Fig. V-13. Method of dealing with salts of volatile acids. (*c*) Micropipette. Martin (442).)

tarded, as well, probably as substances with highly polar groups or acidic groups. With a stationary phase loaded with hydroxylated compounds hydrogen acceptors (bases of all kinds) are likely to be retarded, as are also substances with highly polar groups. An aromatic stationary phase is likely to show special properties because of the possibilities of π-complex formation, and of the polarizability of the aromatic π electrons.

Suggestions for choosing stationary phases can be derived from the Table V-1.

VIII. PRACTICAL CONSIDERATIONS

The method of gas partition chromatography requires relatively simple apparatus. The method can be rapid because of the low viscosity of the mobile phase. It is also particularly easy to recognize the zones as they emerge from the column. For these reasons this is very probably the method of choice for compounds that are sufficiently volatile. James and

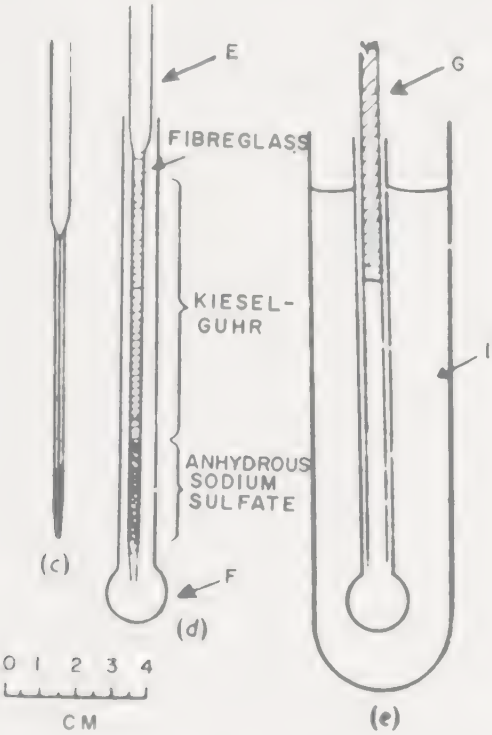


Fig. V-13. Method of dealing with salts of volatile acids. (*c*) Micropipette, (*d*) Micropartition column, *E*, in a test tube *F*. (*e*) End of gas chromatography tube, *G*, in heating bath, *I*. See text. (After James and Martin (441).)

Martin (442) say that in time this method should replace that of analytical distillation.

If the volatile substances are worked up in the form of salts, to restrict their volatility, it is a simple matter (441,444) to convert to the free acid or base as the material is transferred to the column. The salts of volatile acids are converted to the free acids by a method described by Martin and James (441) (Fig. V-13):

"In most biological experiments the volatile fatty acids are isolated as their sodium salts, and a simple method of obtaining the acids free from water is necessary before they can be applied to the column. The presence of even a small percentage of water in the fatty acid sample is sufficient to upset the separation, presumably owing to azeotrope formation.

"The method adopted consists in drawing up a small sample of an aqueous solution of the sodium salts (30 μl. of an approx. 2N solution) into a micropipette (Fig. V-13) that has been pretreated with a silicone (by washing with a 1% (w/v) solution of DC1107 in chloroform followed by heating at 140° for 30 min.), to render it unwettable by water. A small bubble of air is drawn in and an aqueous solution of orthophosphoric acid (60% w/v) is drawn up and the two solutions mixed in the wide part of the pipette. The acidified mixture is then run on to the top of a micropartition column (Fig. V-13), the lower part of which is packed with kieselguhr (Celite 545). The aqueous solution of the acids is taken up by the kieselguhr. Ether (0.6 ml.) is then run through the micropipette on to the column and passed through the column under pressure. The eluate is collected in a long narrow test tube (Fig. V-13), whose diameter is slightly greater than that of the chromatogram tube. When all the ether has been collected the chromatogram tube is removed from its vapour jacket and inserted in the test tube so that the end of the column is just above the surface of the ether. The test tube is immersed in warm water and air is drawn through the column until all the ether has evaporated. The test tube is then immersed in a heating bath (100° for acids up to valeric, 150° for acids up to dodecanoic acid) and air is drawn through the column for 15 min. The acids move up into the column and condense there. At the end of this time the column is replaced in the vapour jacket, the nitrogen gas turned on and the separation carried out as already described."

James, Martin, and Smith have described how the salts of bases may be worked up (444).

The **quantities of materials** that are used are small, of the order of 0.3 to 10 μg. equivalent of each component of a mixture. However, the method can be scaled up considerably. Dimbat *et al.* (232a) report a 4.2 cm. I.D. column, 290 cm. long, that gave almost as efficient separation as a 0.7 × 195 cm. column.

The **time required for an analysis** may be quite short. For example, for a total load of 2 mg. of aliphatic hydrocarbons from n-pentane to n-octane (Fig. V-6) the time required was 34 min. Other examples are given in Figs. V-7 and V-8.

The sensitivity of the method is such that by titration 0.02 mg. of

acetic acid can be detected, and 0.3 μg. equivalent of amine. With the gas density balance of James and Martin, $^1/_{16}$ μg. of amyl alcohol per milliliter of nitrogen can be detected (1 molecule of amyl alcohol in 50,000 of nitrogen).

Recoveries and reproducibility are said to be excellent. For example, James, Martin, and Smith (444) found recoveries of amines to be approximately theoretical, and reproducibility to be $\pm 4\%$ under the conditions described above for working with amine salts.

The range of compounds to which the method is applicable is limited only by volatility. The method is said to be applicable to all volatile substances up to those that can be distilled at a few millimeters pressure of mercury (442). Nonvolatile acids can be converted to methyl esters with increase in volatility. Aromatic hydrocarbons and amines, pyridine homologs, halides, esters, alcohols, ketones, cyanides, ethers, and other compounds have been worked with (see Table V-1).

IX. PRECAUTIONS

Fairly obvious general limitations are set to the method. The method may not be useful with substances that are heat-sensitive: that decompose, isomerize, or otherwise react (for example, by transesterification or transaldolization) unless these reactions are slow. It is useful to use metal tubes for the columns for better heat transfer and more ruggedness. They may be useful with paraffins; but catalytic effects should be expected. Even stainless steel may react with aliphatic acids.

X. ESTIMATION

The method is an extremely powerful one for analytical purposes. Its preparative use has not been adequately tested. It should turn out to be valuable for studying interactions between substances and solvents when the latter are essentially nonvolatile and the former volatile (446).

In using the method to its fullest effectiveness many factors not considered in detail here may have to be taken account of: the cooling effect on the column of the addition of a liquid sample, and the possible fractionation of it during its vaporization, for example. The effects of overloading; the proper positioning of the sensing device, whether in the stream of gas or off to one side and connected through diffusion with the main stream; the relative virtues of peak height or base width for quantitative estimation of recorder plots of zones; and so on, cannot be dealt with adequately here, and must be sought in the original literature or in a more specialized place (469b,730a).

Column Partition Chromatography

I. PRINCIPLE

The mixture to be separated is distributed between two liquid phases. One liquid phase is sorbed, possibly producing a gel in some cases, to a *support* packed as a porous deep bed, or column, in a tube. This is the *stationary*, or *supported*, phase. The mobile phase flows in contact with it, through the column. Separation depends on the distribution of the components of the mixture to different ratios between the mobile and stationary phases (616). Those components that interact more with the stationary phase are retarded with respect to the ones that interact less with the stationary phase.

II. CLASSIFICATION

It is sometimes difficult to distinguish **column partition** chromatography from **adsorption** chromatography and when this is the case the distinction is not important to make. If in adsorption chromatography a component of the developer were to be sorbed to the extent that a multilayer were produced of sufficient thickness to behave as a solvent for components of the mixture, then the conditions for partition chromatography would exist.

The similarity of column partition chromatography to paper chromatography (Chapter VII) approaches an identity in all aspects except those of apparatus and physical appearance.

One major difference is that whereas in column chromatography a tube mechanically sets a limit to the dimensions of the column parallel to the direction of flow, in paper chromatography, this limit is set by the surface forces of the system: the surface tension of the mobile phase. Two other procedures may well be classed with either column or paper partition. These are the chromatostrip and chromatobar procedures and the chromatopile and chromatopack. In both of these types the support is arranged as a bed, and so is like the column type. However, both are like paper chromatography in that the outer limits of the mobile phase are set by surface forces of the developer, and not by the walls of a tube.

III. APPARATUS

The apparatus consists of a tube within which the bed that comprises the stationary phase and its support is formed (as in Fig. II-1). In general, the following considerations apply. For a given mass of stationary phase and support, the deeper (longer) the bed and the smaller the diameter of the tube, the better the resolutions that will be obtained. This is because, at least up to a point, the more opportunities that the molecules of the mixture have to move into the stationary phase (to be retarded) and back to the mobile phase, the more opportunities there are for differences between the components of the mixture to show up. Such opportunities are increased with length of the bed or column, and are not appreciably affected by diameter. A volume element of solution passes over more stationary phase in a long than in a short column. This is a factor of the greatest importance. Also, it is at the front and rear faces of the zones that irregularities in flow and packing cause the distortions that make zones difficult to distinguish and to separate. In a narrow column, the area of these faces is small, and the regions where irregularities may occur less important relative to the total amount of material in the zone, than would be the case (given the same amount of adsorptive) with a shallow, wide bed, with shallow zones of wide diameter. Opposed to this is the fact that the rate of flow of solvent through a bed is related in a linear way to the reciprocal of the column length, for a given pressure-difference between the top and bottom of the column (and with a given column length, linearly with the pressure-difference) (437, 541,544). LeRosen demonstrated this and showed it to follow from Darcy's law (673) (see Fig. VI-1). Thus in practice the two factors must be balanced, one against the other, using columns that are long and narrow enough to ensure good separation, but not so long as to cause impracticably slow flow of mobile phase, through the column.

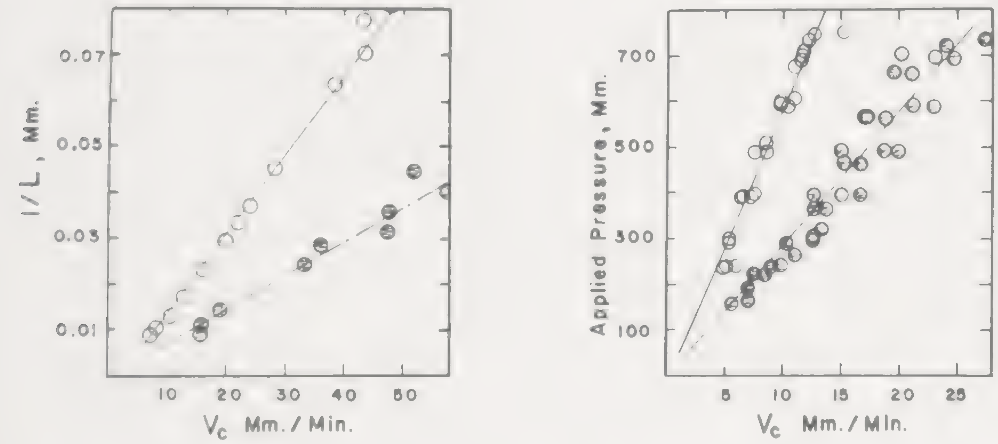
Two other factors, at least, must be weighed here. To some extent, other things being equal, the supporting material, or adsorbent, in a longer column can safely be of a larger average particle size than would give good results with a shorter column. Further, if the mobile phase flows too slowly, diffusion may begin to assume undue importance. It is likely that the flow could be so slow that fuzzy boundaries would be shown by zones of materials which on faster flow would show sharper boundaries. (See Chapter VIII, Section IV, for a thorough discussion.)

The balancing of these considerations led to the column dimensions described in Section VI.

A device may be used to combine the virtues of wide and of narrow columns (357,593). The bulk of the stationary phase is utilized in a wide

column. Below this is set a short narrower column, and below this, perhaps, a short still narrower one. As development proceeds, the fronts emerge from the wider column into the narrower one. The effluent from the wider column is mixed in the space where one column is joined to the other, and then passes to the narrower column, where the fronts are sharpened. Thus in favorable cases the fast-flowing larger column makes the separation rapidly, and the fronts are sharpened in the narrow, but short, columns placed at the exit of the larger one. This technique is not much used with partition columns (but see Chapter VIII, Section VI.2).

Important parts of the apparatus are the devices used for **collecting** fractions and for analyzing them. In some separations indicators serve to mark the zones; in others the effluent from the column is collected systematically and analyzed, then a diagram is plotted to show which fractions contain separated zones of material (see Section VI and Chapter XI).



(*a*) (*b*)

Fig. VI-1. Relations between flow rate of mobile phase and column length and applied pressure. The data are for adsorption columns packed as indicated in Section VI. (*a*) Relation between flow rate (V_c) and column length (L). (*b*) Relation between flow rate and pressure. ○, Benzene–silicic acid. ● Benzene–lime. (From LeRosen (544).)

IV. STATIONARY PHASE

The stationary phase is a liquid that is immobilized on a support. Stationary phases fall into two classes: the more usual, hydrophilic ones; and the "reversed phase" type.

The **support** should preferably be inert to substances being separated, that is, it should not adsorb them. This requirement was stated in their first paper by Martin and Synge (616). These authors, separating

acetylated amino acids on a column of silica supporting an aqueous phase, found that when the developer (mobile phase) was pure chloroform the acids moved very slowly, but that addition of alcohol to the chloroform (0.5 to 1% of ethyl or *n*-butyl alcohol) caused ready movement with separation. The slow movement was laid to adsorption of the acetylated amino acids to the silica. The effect of the alcohol was thought to be due to displacement or elution of these acids from the silica by the more strongly adsorbed alcohol.

A great many substances have been utilized as supports for the liquid phase: silicic acid, diatomaceous earth (kieselguhr, Celite, Dicalite, etc.), powdered cellulose, starch (various kinds, as potato, corn, rice), powdered glass, rubber latex on cellulose. These materials have been used to support polar as well as nonpolar liquids. Thus kieselguhr has been used to support aqueous phases of various kinds. It has been treated with silicone and used to support nonaqueous phases (see Table VI-2).

The stationary or supported liquid phases have been of many kinds, varying in polarity from water to paraffin hydrocarbons (616). A general discussion of the role of the supported liquid and its choice is deferred to Sections VIII and X. Very commonly the supported phase is composed of incompletely miscible liquids, though sometimes mixtures of substances miscible in all proportions, and even pure substances, have been used.

V. MOBILE PHASE

In general, the mobile phase in partition chromatography is "opposite" in polarity to the stationary phase. Often it is composed of several incompletely miscible liquids. That liquids are incompletely miscible implies that they are different in polarity (402). Sometimes the mobile phase consists of a single pure liquid. In such cases, if partition chromatography is operative, the stationary phase must consist of this liquid too, as mentioned above, but in a state of considerably different activity. A discussion of the choice of mobile phase is deferred to sections VIII and X.

The mobile phase may flow downward or upward through the column. It may move under the influence of gravity, applied pressure, or suction. In all cases best results are obtained if the liquid is degassed before use (613). If the liquid is not degassed, bubbles may appear in the column and destroy the uniformity of the packing.

The columns may be buffered, and they may be heated—uniformly or with a gradient of temperature.

VI. PROCEDURES

1. The Good Chromatogram

A good chromatogram is one in which the components of the mixture are separated cleanly. In frontal analysis, the fronts should be sharply

delineated. In elution analysis the zones should be discrete, with sharp front and rear boundaries, and with spaces containing only mobile phase between them. In displacement analysis the zones should follow cleanly one upon the other. And in all cases, in good chromatograms, the column should run with the utmost uniformity.

2. Packing the Column

Columns may be packed **wet** or **dry**. In general, the wet method is best if only because it normally ensures equilibrium between the stationary and mobile phases.

The supporting material must be coated with the stationary phase. This may be accomplished by mixing the stationary phase into the dried pulverized supporting material; or by slurrying this material with mobile phase from which the stationary phase is sorbed by the supporting material.

To pack a column dry, the following procedure can be used. *This procedure applies both to liquid partition and to adsorption columns.* The chromatography tube is assembled (see Fig. VI-2,*a*). Normally this consists of a glass tube with an indentation at the lower end, or a porcelain plate, or a joint and perforated disk. The lower end should be provided with a stopcock or pinch-clamp to regulate flow. A pad of glass wool or cotton is placed at the lower end of the tube to act as a support for the column, and is tamped down with a dowel, or a cork (or disk of wood, plastic, etc.) on the end of a rod (Fig. VI-2,*b*). This tamper should not fit the tube too snugly, but should be slightly smaller in diameter than the tube. The assembly may now be weighed, so that later the weight of the column can be determined by difference.

The tube is held at a slant in the left hand (by a right-handed person) and a *small* amount of the packing material, support or adsorbent (Chapter VIII), is introduced into it with a spatula, or more conveniently a spoon. The material is shaken down on to the pad of glass wool, and tamped into place. The best way to accomplish this tamping is to hold the tube at a slight slant, and lightly but firmly press the material down with the dowel. Zechmeister makes the end of the dowel or disk slightly concave so that a little more pressure is given along the outside circumference of the column than to the central parts (Fig. VI-2,*b*). A cork is easily made sufficiently concave with fine sandpaper. The tamper should be worked along the circumference of the column by pressing down along the wall of the tube, rotating the tube slightly with the left hand, pressing down again, rotating again, and so on.

After the first little portion of material is in place, another small portion is introduced and packed down. Normally, the packing should not be done with much force; with enough only to give a firm column. Here a little practice is required and a few trials. The packing procedure is continued until the previously weighed amount of material has been introduced, or until a column of sufficient length has been built up (Chapters

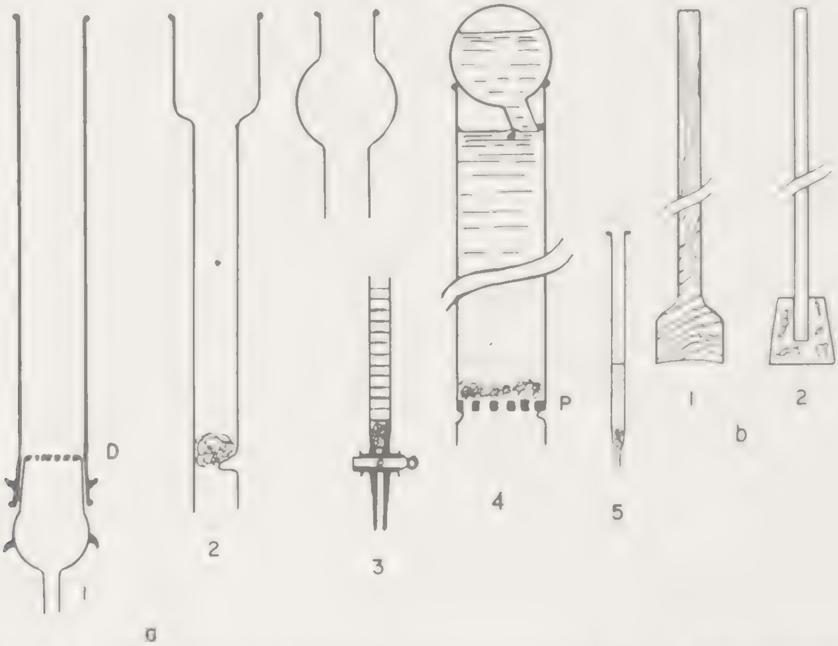


Fig. VI-2. Some apparatus for column chromatography. (*a*): 1, Straight tube, with standard taper joint and porous or perforated supporting disk, *D*. Can be obtained in several sizes (see Appendix). 2, Simple tube with reservoir on top. An indentation supports a wad of glass wool, or cotton. May be equipped with a stopcock, or a rubber tube and pinch-clamp. 3, An ordinary burette, with a wad of glass wool above the constriction serves conveniently, and is already calibrated. Other tubes can be marked by pasting a strip of graph paper along them. 4, Wide tube, with perforated porcelain plate (*P*) (a Witt or Gooch plate) resting on a constriction, or on indentations, and with a constant-level reservoir at the top. 5, A medicine dropper with a column in it. (*b*): 1, A wood dowel with slightly concave end. 2, A cork on the end of a rod. Can be made concave by rubbing with fine sandpaper.

VIII, XV). The top of the adsorbent is then stabilized by covering it with a disk of filter paper, or a pad of glass wool or cotton (tamped down). A porcelain disk or layer of glass beads is then added to weigh down the pad.

It is essential that the column be packed in quite small portions. The extra time and effort required to pack a column well, in small portions,

represents a small investment only when the entire procedure is considered. A poorly packed column will show "pressure-rings" when the mobile phase passes down it (Fig. VI-3,*a*). These are places where the well-pressed-down top of a portion of filling is marked off from the lower face of the next higher portion of filling. This occurs when too large a portion of material is introduced into the column at once, so that the pressure applied at the top of it does not get transmitted in an effective way (slipping of the particles into a compact arrangement) to the lower portion, which remains more loosely packed.

A properly packed column permits even flow of mobile phase, which

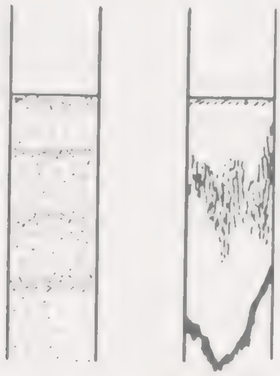


Fig. VI-3. Poorly packed columns. (*a*) The column shows pressure-rings when wetted with liquid. (*b*) This column is operating poorly. It shows irregular zones. May be due to channeling, uneven heating effects, gas bubble development, uneven packing, and probably other causes, alone or together.

advances down the column at a regular pace. Normally, as the front of the mobile phase moves down an initially dry column which it is wetting, the velocity of flow decreases. This is partly due to friction factors, as the liquid has to pass down an increasing length of column. It may be affected by swelling of the packing, if this occurs. Heating effects (heat of wetting) may change the viscosity of the mobile phase, usually, but not necessarily decreasing it. At any rate, after the mobile phase has wetted the entire column, the velocity of flow usually becomes constant for a given pressure-head.

LeRosen (541) has studied some of these questions and has defined certain parameters by which column packing may be characterized.

S = length of the column containing one unit volume of solvent divided by the length of the tube (above the column) required to contain the same volume of solvent.

V_c = rate of flow of mobile phase through the column when a state of constant flow has been reached measured (mm./min.) by the movement of the meniscus of liquid in the tube above the column.

The uniformity of packing of a column can be estimated by measuring S in different regions of the column. LeRosen (541) packed columns by slowly pouring the adsorbent calcium hydroxide into the chromatography tube (dimensions 17 × 250 mm.) under water-pump vacuum, with vigorous tapping of the tube to aid settling of the adsorbent, and final firm tamping of the column. Such columns showed average S values of 1.97 for the top third to 1.79 for the bottom third. There was no consistent relation between S and V_c, he found. The value of V_c varied from column to column. In 14 of 15 cases, it lay between 6.3 and 8.2; in one it was 10.3. He suggested that V_c depends on particle size, shape, surface character, and nature of the mobile phase, as well as other factors (Fig. VI-1). He found a reasonable value of V_c to be about 5 to 15 mm./min. (In the case of the calcium hydroxide these values seemed to be associated with particles of 5 to 15 μ size.)

Wet packing methods are illustrated below.

3. Examples of Entire Procedures

A. USE OF SILICA

Martin and Synge (616) prepared a column by adding to a purified commercial silica "70% w/w of a saturated solution of methyl orange in water. The solution is added to the gel at once and thoroughly mixed in. A dry pink powder results, which barely adheres to the walls of the vessel in which it is mixed." A more reproducible supporting material and supported phase was prepared as follows: "Commercial waterglass (140 Tw) is diluted with 2 vols. water, and 10N HCl is stirred in, with methyl orange as internal indicator. After standing several hours (more acid being added as required) it is filtered and washed with distilled water on a Buchner till washings are free from indicator. The gel is allowed to age, wet, for 1–2 days on the filter, and after further washing dried at 110°. For use in the chromatogram 50% w/w of indicator solution is added to the gel." To form the column, "An amount of this material containing 5 g. of silica is then suspended with stirring in about 35 ml. of chloroform saturated with water and containing 1% v/v of n-butyl alcohol. At this point the colour of the gel changes from pink to yellow. The suspension is poured into a chromatogram tube (int. diam. 1 cm.; length 30 cm., furnished at its bottom with a double layer of filter paper mounted on a perforated silver plate). The gel packs down as the chloroform flows out at the bottom of the tube; being lighter than

the chloroform it floats in it. When it has packed down to its final position, the top surface appears to be dry and if evaporation is prevented, no further chloroform leaves the column, capillary forces preventing the entry of air. The gel does not float up again when fresh chloroform is carefully added at the top of the column. The solvent emerging at the bottom of the column is almost free from indicator, the methyl orange being firmly held in the aqueous phase."

This column was used to separate *N*-acetyl amino acid. "The substances for analysis are dissolved in a little chloroform–butyl alcohol, and this solution is carefully added to the top of the column by allowing it to run from a pipette down the side of the chromatogram tube. When this addition and any washings have drained into the gel, the chromatogram is developed by adding fresh solvent at the top of the column.

"The position of the acids is revealed by the indicator, which turns from yellow through orange to pink. As development proceeds the single pink band at the top of the column resolves into constituent bands which move down the column at characteristic rates."

This type of procedure is quite general. Special methods have been described for preparing uniform silica (337,371,434,945) but commercial silicas seem often to be quite satisfactory. As the zones of separated acetyl amino acids leave such columns they are collected separately, being clearly distinguishable because of the color changes as they pass along the indicator-loaded column.

B. USE OF KIESELGUHR (DIATOMACEOUS EARTH, CELITE, SUPERCEL, DICALITE)

Martin (613) has given explicit directions for preparing kieselguhr columns. "Kieselguhr columns cannot be packed as a slurry in the mobile phase as silica gel can, by simply pouring it into the chromatogram tube. The kieselguhr, after mixing with half its weight of stationary phase (with a spatula in a beaker), can be packed with a ramrod into the tube. This method gives indifferent columns. A much better method is to slurry the mixed kieselguhr and stationary phase with enough mobile phase to give a creamy consistency. The slurry is poured into the tube and thoroughly homogenized by a few rapid strokes of a perforated disk, which is mounted by its centre on a long wire handle. The disk should fit the tube closely. The disk is brought to within about an inch of the bottom of the tube and then moved slowly downwards. This causes the kieselguhr to pack beneath it. Rapid strokes followed by a slow packing stroke are repeated until the whole column is packed. If the kieselguhr and stationary phase mixture is heavier than the mobile phase, a funnel with a wide tube attached to the top of the chromatogram tube will save constant

refillings. If the mobile phase is the heavier a cork in the top of the chromatogram tube with a hole in it just large enough to provide easy passage for the handle of the disk is a useful aid. The columns when packed are robust and free from gas. Excess liquid may be poured off without fear of disturbance, and their regularity is at least as good as a precipitated silica column, and their efficiency in terms of a height of equivalent theoretical plate is probably higher. There is more resistance to flow of solvent through the column and a few pounds pressure is usually desirable. Whether vacuum or pressure be employed it must be emphasized that the solvent must be kept degassed if trouble with the separation of gas in the column is to be avoided. With thoroughly degassed solvents, gas always disappears from the column even if originally present.

"The size of the holes in the perforated disk should be governed by the interfacial tension between the phases. Where this is high $^{1}/_{16}$ in. holes are satisfactory, when low smaller holes should be used, or packing becomes extremely slow. The interfacial tension probably governs also the proportion of stationary phase to kieselguhr that is satisfactory. When the interfacial tension is high more than one of stationary phase to two kieselguhr may be used."

The remainder of the procedure and the running of the column follow the patterns illustrated in the first example and the next.

C. USE OF STARCH

Stein and Moore (875,877) have given very complete directions for the preparation of starch columns. The method is not commonly used for the separation of free amino acids at present, having been replaced by these authors' method using ion exchange resins. One of the simpler separations with a starch column is illustrated in Fig. VI-4.

D. USE OF A CELLULOSE COLUMN (FOR AN INORGANIC SEPARATION URANIUM)

Pollard and McOmie (739) have described many techniques for inorganic chromatography. To prepare cellulose columns the following can be used.

Finely divided cellulose can be prepared by boiling scraps of filter paper for 2 to 20 min. in dilute nitric acid (5 ml. conc. nitric/100 ml.). The resulting pulp is washed with water, then alcohol, then ether to remove excess nitric acid. Alternatively, (144) filter paper tablets may be used, or commercial cellulose powders. These authors say (739):

"The glass tube in which the cellulose powder is packed can first be rendered water-repellent on the inner walls by either (a) shaking a

small amount of dichlorodimethyl silane in the tube until the whole surface is wetted with it, draining the tube, and finally washing with alcohol before use, or (b) treating it with a solution of *Dow Corning Fluid 200* in carbon tetrachloride, draining and then heating the tube to 250°C. The latter is preferred because there is quite an unpleasant smell with dichlorodimethyl silane, and also because the silicone fluid is now available commercially. It is claimed that this treatment of the tube prevents the creeping of solvent between the cellulose column and glass. If fluorides are absent the surface will remain water-repellent for some time. While this treatment is considered very necessary by some workers, others find that with careful packing of the tube no such precautions are required. It is possible that it is more essential with some eluting solvents than

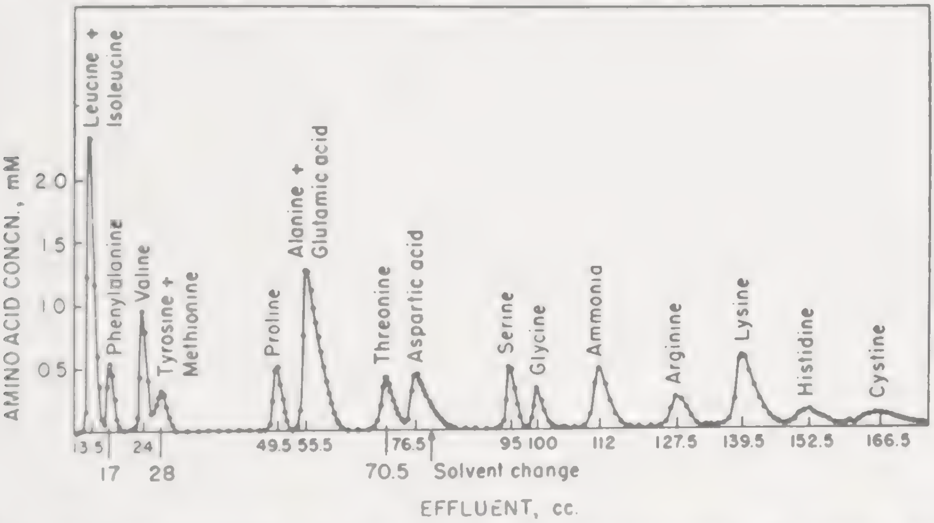


Fig. VI-4. Chromatographic fractionation of a hydrolyzate of bovine serum albumin (34). The sample corresponded to about 2.5 mg. of protein, and was separated on a 0.9 × 30 cm. column of starch. The first developer mixture was 1:2:1 1-butanol:1-propanol:0.1 *N* HCl. At the point marked, the developer was changed to 2:1 1-propanol:0.5 *N* HCl. (From Stein and Moore (877).)

with others. Recently polythene tubes have been used with solvents likely to attack glass, e.g., hydrogen fluoride" (144,145).

The column may be formed by gravity packing. The tube is half filled with mobile phase, and the cellulose is introduced in small amounts with enough stirring to disperse the powder. After it has settled, another small batch is introduced and so on until a column of sufficient height has been prepared. This is the method of choice when the mobile phase is fairly volatile.

Alternatively, the powder is slurried in the mobile phase (some workers grind them together in a mortar). The mixture is put in portions into the

tube and pressure is applied to drive out excess liquid. When the powder does not pack down any further, but before the top layer becomes dry, the pressure is released, another lot of slurry is introduced, and the process is repeated until a column of sufficient height is produced.

As with all other columns, the cellulose column must not be permitted to run dry. If it dries, it shrinks away from the tube and leaves channels. A well-packed column flows evenly, and gives zones with regular, well-delineated boundaries.

For the determination of uranium in low-grade siliceous ores, monazite sands, and so on, a cellulose column (146) and suitable mobile phase serve to retain many cations but allow the uranium to pass. Pollard and McOmie (739) say: "Use is made of the well-known solubility of uranyl nitrate in ether and other organic solvents. Simple extraction without paper chromatography does not always yield a pure product, but with chromatography, providing certain conditions are observed, the uranium can be obtained free from other metals. Cellulose columns enable larger quantities to be used and thus offer advantages over the paper strip method, particularly where only a small percentage of uranium is present in the material to be analyzed. For practice, a synthetic mixture containing Fe, Zn, Mn, Cr, V, and Cu is suggested in this chapter, but successful methods for the analysis of siliceous ores and monazite sand have been developed (146).

"With the solvent mixture recommended—ethyl ether containing 5 per cent v/v nitric acid—a very large number of cations hardly moves at all. If the ether contains peroxide, vanadium moves rapidly as a pink peroxyvanadium compound, but if a reducing agent such as ferrous sulfate is present the pink compound is reduced to an immobile vanadium salt. Metals which move and might cause some interference are gold, mercury, selenium, arsenic, antimony, bismuth, cerium, thorium, zirconium, scandium, tin, the platinum metals, and molybdenum. Reference should be made to the original paper for details of how to overcome such interference. The presence of certain anions like phosphates, sulfates, halides, can also cause difficulties.

"Prepare columns of cellulose (5 to 8 cm. long) by the gravity method . . . [see above] and transfer the prepared solutions (either synthetic or from ores) absorbed in a suitable quantity of cellulose (2 g. of cellulose for 10 ml. of the synthetic solution) to the top of the column.

"(i) Elute with ether-nitric acid mixture until 150 ml. of eluate has been collected. (ii) If the solution is from siliceous ores, or monazite sand, elute as above but to the final aqueous residues add 5 ml. of sulfuric acid and 5 ml. of perchloric acid, evaporate until fuming commences. Complete the estimation by a suitable method."

E. USE OF A REVERSED PHASE COLUMN

The first suggestion of reversed phase-chromatography phases was made by Boscott (95). A column of this type was described by Boldingh (90) for the separation of C_8 to C_{18} fatty acids. He used a column of powdered rubber. The stationary phase was benzene, and the mobile, aqueous methanol. However, the conditions of operation were fairly critical (limitation of temperature of operation to 21° to 23°C., for example), and what appears to be a more generally applicable method is given as the example below.

Howard and Martin (425) were able to separate and analyze mixtures of lauric, myristic, palmitic, and stearic acids (*ca.* 2 mg. of each) on a reversed phase column described below. They report that the method appears applicable to acids even above C_{22}. The stationary phase was an organic one, supported on kieselguhr, and the mobile phase a more polar, aqueous one. The preparation and running of the column, and some results, are taken from Howard and Martin as follows.

The kieselguhr, Hyflo Super Cel, was dried at 110°C. It was cooled, and let stand in a desiccator over dichlorodimethyl silane. Alternatively, the dry kieselguhr was stirred while passing through it dry air that had been drawn through dichlorodimethyl silane. The treated kieselguhr should all float when shaken with water. It was washed with methanol until no more acid (bromothymol blue indicator) was removed, and dried at 110°C.

"The standard columns used were 12 mm. in diameter. The mixture required for preparing the more polar phase, e.g., 60% (v/v) acetone in water, was equilibrated with the less polar solvent, e.g., liquid paraffin. The relative proportions of the two phases equilibrated seemed to be immaterial. After equilibration, the two immiscible phases were allowed to separate and drawn off for use when required. At first, columns were packed with 4 g. of kieselguhr and 3.8–4.0 ml. of the less polar phase, but better results were obtained by using 9 g. kieselguhr and 8 ml. of the less polar phase, giving a column 20 cm. long. The stated amounts of kieselguhr and less polar phase were stirred together in a beaker until a homogeneous powder was obtained; this was suspended in the more polar phase and the mixture was stirred by hand to remove lumps. The resulting slurry could not be packed into a column by gravity, but good columns were obtained by using a plunger. This was a perforated stainless steel disk attached at its center to a long stainless steel rod. A portion of the stirred slurry was poured into the chromatograph tube and homogenized by rapid up and down movement of the plunger, which should fit the tube closely. The resulting fine suspension was then packed by slowly

moving the plunger downwards. This procedure was repeated until all the material was packed. When the interfacial tension of the phases is low, packing cannot be effected by the plunger until the suspension has settled somewhat. It was found necessary to homogenize the kieselguhr-paraffin mixtures in a separate tube before starting to pack the column, and this was desirable on all occasions when the material was particularly lumpy. If air is inadvertently included during homogenizing, it must be removed by cautious evacuation prior to packing. Some of the solvent systems tested gave columns which tended to lose some of the stationary phase during use; aqueous methanol–$CHCl_3$ columns were particularly unstable, whereas aqueous acetone–medicinal paraffin columns have been used 14 times without changing their characteristics. The fluctuation of the room temperature during 24 hr. may so change the mutual solubilities of the less stable systems that the characteristics of the column alter. It is then advisable to maintain the column at a constant temperature if it is to be kept overnight. A constant temperature of 35° was normally used for convenience and because the increased solubility of fatty acids at that temperature was an additional advantage. There is no reason to think that other temperatures are unsuitable since good results have been obtained at room temperature when the column was used for a few hours only. The solvents were run down the columns at the rate of 20–50 ml./hr., but slower rates, down to 10% of this speed, give better separations. Such a slow rate is not, however, necessary in most cases.

"*Loading*. The acids to be analyzed were dissolved in the solvent to be used for the development and the solution (approx. 2 mg. of each individual acid in approx. 2 ml. of solvent) was added to the top of the column. This solution was allowed to soak in and was washed down with two ml. portions of the solvent. . . . Fatty acids readily form supersaturated solutions. If these crystallize when loaded on to the column, the solid acid dissolves slowly and causes a steady trickle of acid down the column. High blanks and low recoveries result. When this crystallization is avoided, the recovery is in the range of 95–100%. If a mixture of fatty acids of very different chain length is encountered it may not be possible to dissolve it in a solvent suitable for separating the shorter-chain acids. In this case a solvent suitable for the longer-chain acids must be employed and the first runnings from the column must be refractionated on the appropriate column.

"*Siphon*. The siphon *A* (Fig. VI-5) may be adjusted to deliver various volumes by moving the plunger; 2 ml. was normally delivered.

"*Titrations*. Titrations were carried out under N_2 using cresol red or, in later experiments, bromthymol blue as indicator. It was found convenient to dissolve the indicator in the developing solvent. Standardized

0.01 N-KOH in methanol was used for the titrations and stored in a dark internally waxed bottle. A 2 ml. microburette graduated by 0.01 ml., was used to deliver the alkali to the titration cup. The alkali was protected at all points from atmospheric CO_2. The accuracy and speed of the titrations was improved by using the apparatus shown in Fig. VI-5. Light from the lamp is led to the titration cell (*B*) by a tube (*C*) filled with $CHCl_3$ and the colour is observed by looking endwise down the cell.

A capillary tube at the lowest part of the cell admits a nitrogen stream to circulate the liquid during the titration, and drains the cell when the tap *D* is opened. With this apparatus the colour change in a 6.5 cm. depth of liquid can be observed with a total volume of 2 ml. The approach of the end-point is heralded by a flickering colour change. With bromthymol blue, the end point was taken to be the first persistent green colour. Attempts were made to follow the behaviour of the columns by a conductometric method, but were abandoned because of erratic behaviour of the electrodes and the satisfactory nature of the titration method.

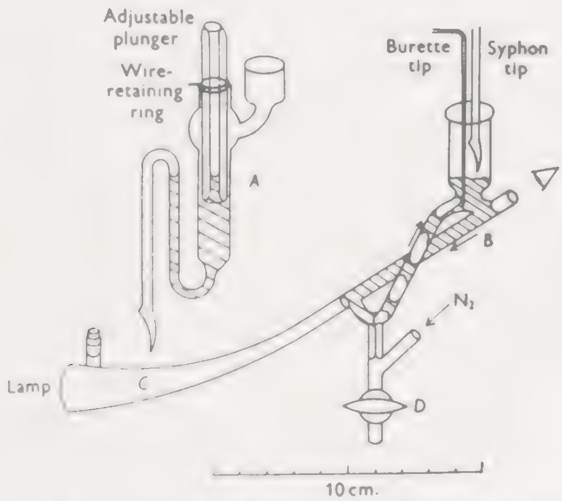


Fig. VI-5. Diagram of apparatus. *A*, Adjustable siphon; *B*, titration cell; *C*, tube filled with $CHCl_3$ to transmit light to titration cell; *D*, tap through which to empty cell. (After Howard and Martin (425).)

Solvent systems. The most satisfactory systems tested were aqueous acetone-medicinal paraffin, and aqueous methanol–*n*-octane or cyclohexane. The proportion of water in the solvent was chosen to give the most rapid development consistent with good separation. Increasing the proportion of water decreased the rate of development and improved the separation but increased the width of the band. The most satisfactory procedure with a complicated mixture of acids was to change the solvent

after the shorter-chain acids had emerged. The change was to a solvent containing a smaller proportion of water. The points at which such changes should be made can be decided readily on the basis of titrations."

Table VI-1 shows results obtained by Howard and Martin with various acids and solvent systems, and Fig. VI-6 shows the course of a typical separation. With unsaturated acids, of the same chain length, increasing

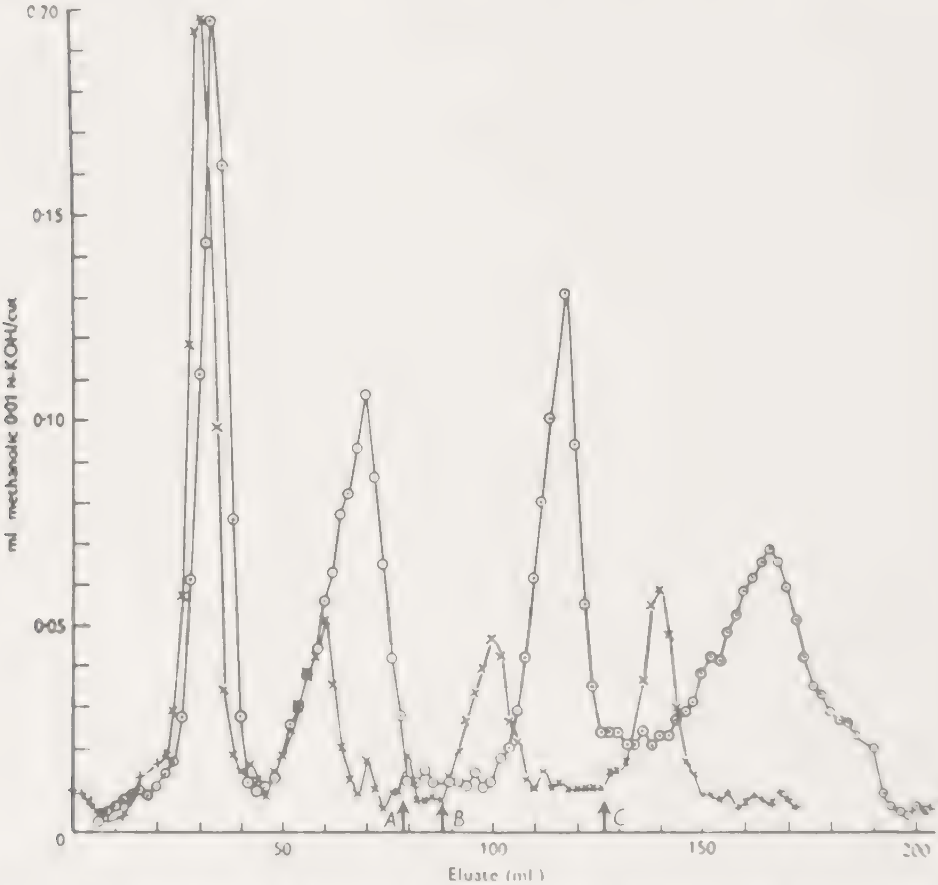


Fig. VI-6. Separation of a mixture of lauric, myristic, palmitic, and stearic acids (X), using 70% and 80% (v/v) aqueous methanol saturated with *n*-octane as the moving phase and *n*-octane saturated with 70% (v/v) aqueous methanol as the stationary phase. Moving phase changed from 70% to 80% aqueous methanol-*n*-octane at *B*. ⊙, using 55%, 68%, and 55% (v/v) aqueous acetone as the stationary phase. Moving phase changed from 55% to 68% aqueous acetone at *A* and to 70% aqueous acetone at *C*. Ambient temperature 35°C. (After Howard and Martin (425).)

the degree of unsaturation increases the rate of movement down the column, that is, increases the distribution into the more aqueous, mobile phase. *Trans* acids are retarded more than *cis* (elaidic, oleic; erucic, brassidic) and thus behave in a "less unsaturated" manner.

TABLE VI-1
Behavior of Fatty Acids in Reversed-Phase Partition Columns[a]

Solvent system	Lauric acid			Myristic acid			Palmitic acid			Stearic acid			Oleic acid			Dihydroxy-stearic acid			Elaidic acid		
80% methanol–cyclo-	—	—	—	34	48	60	70	90	110	—	—	—	—	—	—	—	—	—	—	—	—
hexane	—	—	—	28	40	52	60	78	100	—	—	—	—	—	—	—	—	—	—	—	—
60% methanol–n-octane	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	10	15	24	—	—	—
70% methanol–n-octane	22	34	44	48	70	80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	22	32	40	48	68	78	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
				52	76	94	110	192	220	—	—	—	—	—	—	—	—	—	—	—	—
80% methanol–n-octane	—	—	—	24	32	40	42	56	78	90	116	130	—	—	—	—	—	—	—	—	—
				24	36	44	50	64	74	—	—	—	—	—	—	—	—	—	—	—	—
50% acetone–medicinal paraffin	14	34	48	36	78	94	—	—	—	—	—	—	—	—	—	8	12	30	—	—	—
55% acetone–medicinal	20	32	44	48	60	68	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
paraffin	14	26	32	32	44	60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
				32	46	54	68	96	110	—	—	—	—	—	—	—	—	—	—	—	—
60% acetone–medicinal	—	—	—	30	40	52	62	80	90	—	—	—	—	—	—	—	—	—	—	—	—
paraffin	—	—	—	20	40	50	50	80	96	—	—	—	—	—	—	—	—	—	—	—	—
65% acetone–medicinal	—	—	—	10	24	45	32	52	70	—	—	—	—	—	—	—	—	—	—	—	—
paraffin	—	—	—	16	28	44	—	—	—	—	—	—	34	54	70	—	—	—	40	56	90
70% acetone–medicinal	—	—	—	—	—	—	—	—	—	40	60	76	—	—	—	—	—	—	—	—	—
paraffin	—	—	—	—	—	—	—	—	—	36	58	72	—	—	—	—	—	—	—	—	—

The numbers indicate the start, peak, and end of the zones as the total volume (ml.) of eluate collected at these points. These figures were obtained with the standard column 20 cm. long and 12 mm. in diameter made in the way indicated in the text with the solvent system quoted in the left-hand column. Solvent systems are defined as % (v/v) of the first-named substance in aqueous solution equilibrated with the second-named substance.

[a] From Howard and Martin (425).

The above examples cover by implication all types of the manipulations that may be required in column chromatography, whether organic or inorganic mixtures are separated; whether qualitatively or quantitatively; whether by conventional or reversed phase operations. In order to extend the description to as wide a range of compounds as possible, Table VI-2 has been drawn up.

VII. RECOGNITION AND CHARACTERIZATION OF ZONES

Chapter XII is devoted to a discussion of the recognition of zones. In the following chapter, VII, Sections XIV and XV, there is given a discussion of the factors that affect the sharpness of zones and which affect recognition. Many of the effects discussed there can be related to problems encountered with columns, and it is therefore unnecessary to repeat them here, as an example will show.

In that section (Chapter VII, Section XV) it is pointed out that several zones have been observed from a single substance, and it is discussed how these multiple zones may have been produced. One cause is a gradient in the developer due to frontal analysis, or other concentration change. Such multiple zoning was observed in partition columns by Smith(864). He observed that when a prepared kieselguhr column was treated with butanol–water developer two and sometimes three zones could be observed with a single pure solute (vitamin B_{12b}). He found that if the developer was not fully saturated with water it could remove water from the upper part of the column, thus leaving it unduly dry; if the developer contained suspended water it could deposit this in the upper part of the column, waterlogging it. These changes in water-holding power of the developer could be due to improper preparation or could be induced by temperature changes during a run. The solute, if very hydrophilic (that is, with a low **R** value), then may be more than usually sensitive to change in water content of mobile and nonmobile phases. In the case in question, vitamin B_{12b} is taken up by the column from undersaturated aqueous butanol—it is eluted again by fully saturated butanol. Thus a zone, formed near the top of the tube and subjected to a succession of units of developer with different water contents, would move in part into the water-rich phase whichever it was, thus becoming split into more or less separate zones. Such behavior is fortunately a rare occurrence. The explanations for it, and other zone irregularities and artifacts that affect zone recognition, can, then be derived from the discussion in Chapter VII.

For a given system of stationary and mobile phases, zones in column partition chromatography are characterized by their **R** values. This was

defined and discussed in Chapter IV. The **R** value is a measure of the retardation (and therefore also of the mobility) of the zone. It reflects the interaction of the solute in the zone with the stationary phase relative to its interaction with the mobile phase. It is measured as the ratio of the velocity of movement of the center of density of the zone down the tube to that of the mobile phase. The latter is measured by timing the movement of the meniscus of developer above the column.

No tables of **R** values are given here. They may be found in compilations of the literature. Normally, anyone working with column chromatography will compile his own tables, using known pure substances relevant to his work; and he will compile, also, the more reliable tables of *relative* R values. (See Chapter IV, Section X.)

VIII. FACTORS THAT AFFECT R VALUES. THE RELATION BETWEEN R VALUES AND MOLECULAR STRUCTURE. CHOICE OF PHASES

All these important topics are treated in detail elsewhere. The factors that affect R values are among those that are discussed as factors that affect R_F values, in Chapter VII, Section XIV. The relations between R values and molecular structure are gathered in Chapter XIII or can be derived from the discussion there. The choice of phases is discussed in Chapter XIV in connection with the solution of chromatographic problems. A good deal of information, helpful in choosing phases, can derive directly, or by analogy from Table VI-2.

IX. PRACTICAL CONSIDERATIONS

In column partition chromatography the apparatus can be of the utmost simplicity, and, usually, a tube with a stopcock at the lower end suffices. When colored substances, or acids or bases that can be used with an indicator column, are to be separated the procedure is quite straightforward. With colorless substances it is more complicated. The procedures given for solving problems in Chapters XIV and XV should be followed; otherwise much time and material can be wasted.

Column partition will usually handle larger quantities of material than will paper (59), and when cellulose columns are used the results obtainable rapidly with paper can usually be transferred and scaled up to column size. The elution analysis technique can handle less material per unit of stationary phase than can the displacement technique (611,613).

Column partition chromatography can handle quantities of material down to the limit of detectability. Some idea of capacities and repro-

TABLE VI-2

Examples of Separations Using Partition Columns, Classified According to the Supporting Material and the Substance Separated

Substances	Stationary (s) and mobile (M) phases, and method for observing zones	References
	Silica, Silicic Acid	
Acetyl peptides	(s) aqueous, (M) butanol–chloroform ("B-C"); ethylacetate–water	335
Acids, aliphatic		
C_1–C_4, lower fatty acids	(s) aqueous, (M) butanol–chloroform ("B-C"); bromocresol green on column	264,753,863
C_2–C_8	(s) buffers, (M) B-C; titration	662,813
C_2–C_8	(s) aqueous, (M) B-C, 199:1; indicators on column	280
C_5–C_{10}	(s) methanol, (M) isooctane; bromocresol green on column	690,755
C_{11}–C_{19}	(s) furfuryl alcohol–2 aminopyridine, (M) *n*-hexane; titration	756
C_2–C_{12}	(s) 7.5 ml. aqueous 1*N* NaOH made to 1 l. with absolute methanol, (M) isooctane, then 10% (v/v) isooctane-ether; then ether; bromocresol green on column, and titration	958
n- and *iso*-butyric	(s) methanol, (M) isooctane; bromocresol green on column	752
formic, acetic, butyric, succinic, lactic	(s) aqueous, (M) benzene-butanol; titration	682
Acids aromatic (see also below)	(s) 90% aqueous methanol–0.5*N* sulfuric (9:1), (M) Skellysolve B; titration	71
Acids, dicarboxylic		
C_4–C_{10}	(s) slightly alkaline, aqueous, (M) 5% (v/v) B-C, then 10% (v/v) B-C; titration	958
C_4–C_6	(s) aqueous, (M) 10% (v/v) B-C, then 20%, then 35%; titration	399
C_6–C_{10}	(s) 1 *M*, pH 5.4, citrate buffer, (M) 3% (v/v) B-C, then 5%, 10%, 20%, 35%; titration	399
Acids, keto, as 2,4-dinitro phenylhydrazones	(s) aqueous, (M) series of solutions: 100 ether saturated with water, plus 1, 2, and 3 ml. 95% ethanol equil. with 2.5 ml. 2*N* HCl; self color	540
Acids, *N*-acetyl and DNP amino	See DNP- and *N*-acetyl	
Acids, variety of 1, 2, 3-COOH	(s) 0.5*N* sulfuric, (M) B-C from 5% up to 50%; titration	136

TABLE VI-2 (*Continued*)

Substances	Stationary (s) and mobile (M) phases, and method for observing zones	References
	Silica, Silicic Acid (*continued*)	
aliphatic, aromatic dibasic, tribasic	(s) aqueous, (M) B-C with increasing amounts of butanol; titration	618
Alcohols		
monohydric	(s) aqueous, (M) CCl_4, then $CHCl_3$-CCl_4 1:3, then 1:1; then $CHCl_3$; then $CHCl_3$-acetic acid 9:1; oxidation	209
dihydric, C_2–C_4 glycols	(s) aqueous, (M) B-C 1:4, then 1:1, then butanol; periodate titration	209
Aldehydes as semicarbazones	(s) water, (M) $CHCl_3$ saturated with water; gravimetric	233
Anthocyanidins	(s) dilute orthophosphoric acid, (M) butanol-ethyl ether; self color (s) 10% phosphoric acid, (M), w g. phenol plus $w/2$ ml. toluene saturated with 10% phosphoric acid; self color	870,871
Bile pigments	(s) aqueous, (M) 10% B-C; fluorimetric	466
Copper salts of amino acids	(s) aqueous phase, (M) phenol-$CHCl_3$ (1:1) saturated with water; self color	995
Cytochrome C, purification	(s) 0.15N HCl, (M) chloroform–hexane 1:1; spectrophotometric	654,655
DNP amino acids	(s) aqueous, (M) B-C and others; self color	80,227,633, 731,744, 805,806
	(s) phosphate and other buffers, (M) chloroform, or B-C; self color	80
DNP peptides	(s) water, buffers, (M) B-C, others; self color	807
Grisein (antibiotic)	(s) pH 4.6, 0.1M citrate buffer, (M) 17% phenol in $CHCl_3$ (v/v); self color	506
Hexachlorocyclohexanes	(s) nitromethane, (M) n-hexane; gravimetric	754
Methylated glucoses 2°, 3°, 4°	(s) aqueous, (M) $CHCl_3$, B-C; Molisch test on dried column	61,62,821
N-Acetyl amino acids	(s) aqueous, (M) B-C; indicators on column	82,333-336, 558,906
Penicillins and salts	(s) buffers, aqueous tertiary amine, (M) wet chloroform–ether, wet butanol, others; biological; colored marker	59,91,287, 289,536, 549

Table continued

TABLE VI-2 (*Continued*)

Substances	Stationary (s) and mobile (m) phases, and method for observing zones	References
	Silica, Silicic Acid (*continued*)	
Phenacetin, caffeine	(s) aqueous, (m) diisopropyl ether–chloroform 3:1 (v/v), then chloroform; spectrophotometric	400
Phenol and cresols	(s) aqueous, (m) cyclohexane; spectrophotometric	1028
Porphyrins	(s) water, (m) 20 to 50% chloroform in ligroin, with increasing $CHCl_3$ concentration; self color	570
Pyrrolidone carboxylic acid	(s) water, (m) 17% B-C; indicator	364
	Kieselguhr, Diatomaceous Earth, Celite, etc.	
Acids, aliphatic		
C_2–C_{10}	(s) aqueous 27 to 35 *N* H_2SO_4, (m) benzene, benzene–petroleum ether, others; titration	726,727
C_5 isomers	(s) phosphate buffers, (m) $CHCl_3$ and B-C; gravimetric	134
C_7 acids	(s) methanolic, (m) ligroin methanol; indicator on column	138
variety	(s) 0.5*N* sulfuric, (m) ether, or B-C; titration	730
Alcohols, di- and trihydric	(s) aqueous, (m) ethylacetate or benzene-butanol; periodate titration	683
Cholesterol oxidation products	(s) methanolic or glycolic, (m) cyclohexane sat'd. with 95% aqueous methanol; petroleum ether sat'd. with propylene glycol	556
DNP-amino acids	(s) buffer, (m) wet ether or chloroform; self color	482,722
DNP-peptides	(s) aqueous, (m) wet ethyl acetate; self color	503
Gamma-globulin fractionation	(s) aqueous, (m) organic from 480 g. water, 238 g. 2.5*M* potassium phosphate pH 9.0 buffer, 312 g. butyl cellosolve–ethyl cellosolve 3:2, at −3.1°; spectrophotometric	743
Glycolipid	(s) aqueous; indicator	447
Penicillins	(s) pH 5.5 citrate buffer, (m) ethyl ether-diisopropyl ether 1:1 (v/v); electrical conductivity	443
Pituitary extract (ACTH preparations)	(s) 0.2*N* HCl saturated with isobutyric acid, (m) isobutyric layer; colorimetric, biological	387
Proteins	(s) organic layer, (m) aqueous layer from 83 ml. water, 45.5 ml. butyl	742

TABLE VI-2 (*Continued*)

Substances	Stationary (s) and mobile (M) phases, and method for observing zones	References
	Kieselguhr, Diatomaceous Earth, Celite, etc. (*continued*)	
	cellosolve, 10 ml. 2.5M potassium phosphate pH 7.6 buffer, & others; spectrophotometric	
Ribonuclease	(s) organic layer, (M) aqueous layer from 56% water, 20% ammonium sulfate, 24% cellosolve (w/w/w) & other ratios; spectrophotometric and biological	615
	Starch	
Adenine, guanine	(s) aqueous, (M) butanol–water–ethylene glycol monomethyl ether; spectrophotometric	258
Adrenocorticotropically active peptides	(s) aqueous, (M) 0.1N HCl–propanol–butanol (1:1:1); ninhydrin	88
Amino acids	(s) water, (M) butanol-water and other; ninhydrin	94,539a,648, 649,732, 827,860, 875–877, 905,950
Ammonia and amines	(s) aqueous, (M) butanol–water; titration	305
Pentose nucleosides and nucleic acid	(s) aqueous, (M) butanol–water; spectrophotometric	56
Peptides	(s) aqueous, (M) butanol–water butanol–propanol-HCl, other mixtures; ninhydrin	92,94,283, 303,648, 704,869, 876,905, 907
Purines, pyrimidines	(s) water, (M) propanol–0.5N HCl, 2:1; spectrophotometric	210,211
Ribonucleosides	(s) aqueous, (M) butanol-water; spectrophotometric	766,767
	Cellulose	
Amino acids, α-, β-, γ-	(s) aqueous (M) alcohols; ninhydrin (s) aqueous (M) butanol-acetic acid-water; ninhydrin	81,725,908
Corticosterones, 17-hydroxy-	(s) propylene glycol, (M) toluene; silver nitrate test, and spectrophotometric	25
Gold	(s) aqueous, (M) wet ethyl acetate–nitric acid; electrolytic	465
Keto acids, as 2,4-dinitrophenylhydrazones	(s) ammonia-saturated amyl alcohol (M) gradient, butyl–decyl alcohol,	248

Table continued

TABLE VI-2 (*Continued*)

Substances	Stationary (s) and mobile (M) phases, and method for observing zones	References
	Cellulose (*continued*)	
Keto acids, as 2,4-dinitrophenylhydrazones (*continued*)	increasing in butyl content; spectrophotometric	
Inulin, components of	(s) aqueous, (M) butanol half-saturated with water–ligroin 3:7; hypoiodite	410
Methylated sugars	(s) aqueous, (M) ligroin–butanol 3:2 (v/v); reducing action	423
	(s) aqueous, (M) methyl ethyl ketone–water azeotrope; color reagents	314
Mucilage, linseed, components of	(s) aqueous, (M) butanol–water; color tests and gravimetric	256
Mucilage, slippery elm, components of	(s) aqueous, (M) butanol–ethanol, 95:5, nearly saturated with water; gravimetric	408
Mucin, frog spawn, components of	(s) aqueous, (M) butanol half-saturated with water	295
Phospholipids (crude lecithin)	(s) aqueous, (M) $CHCl_3$ 800 ml., ethanol 200 ml., water 25 ml.; paper chromatographic	70
Rhamnose, arabinose	(s) aqueous, (M) butanol–water containing 1% ammonia; silver nitrate test	422,423
Sugars and derivatives	(s) aqueous, (M) butanol–water; gravimetric	407,409,422, 423
Thymidine, uracil, deoxyriboside	(s) aqueous, (M) butanol–water, or tert. butyl alcohol–pyridine–water (65:25:15), and others; spectrophotometric	224
Xylan, components of, methylated	(s) aqueous, (M) butanol–ligroin 3:7, saturated with 1% ammonia, then butanol; hypoiodite	174
	Glass Powder	
Nitranilines, *o*-, *p*-nitrophenols, *o*, *p*-hyoscine and hyoscyamine	(s) chloroform, (M) wet chloroform; self color	709
	Rubber, Chlorinated	
DNP amino acids	(s) butanol, (M) buffers saturated with butanol; self color	715

NOTE: All alcohols used in mobile phases are normal, e.g., 1-butanol, 1-propanol, unless stated otherwise.

ducibility can be gained from the following figures. Stein and Moore (875) report with a starch column 0.9 × 30 cm., amino acid recoveries accurate to 3% to 5% in individual determinations, with 0.1 mg. quantities of amino acid. The average of several experiments gave recoveries to within ±3% on synthetic mixtures of nineteen components.

X. CHROMATOSTRIPS AND CHROMATOBARS

Kirchner, Miller, and Keller (479,641) have contrived two novel chromatographic columns, one as a strip, the other as a rod, or bar. The chromatostrip is a glass strip coated with a thin, even layer of adsorbent. A mixture of adsorbent, water, starch, and any other additives such as fluorescent pigments was heated till the starch coagulated to give a thick paste, worked with added water to a consistency thin enough to spread and coated evenly on strips of glass. The strips were dried in a forced-draft oven at 105°C. for 15 min., when a thin hard surface of adsorbent was produced. A wide variety of adsorbents could be spread on glass in this manner.

Chromatobars are columns, usually square, formed around a glass-rod backbone for strength, made by pouring an adsorbent, plaster of Paris slurry, into a prepared form and letting it harden.

Both kinds of columns have the advantage that they are easily accessible for spot testing to follow a development process, or for spraying at the end of a run. They are developed by the upward-flow method. They have been used for the investigation of the constituents of essential oils (479,641,642).

XI. ESTIMATE

This is a powerful method of great importance. In general, it is not so rapid nor as convenient as paper chromatography (Chapter VII). The convenience of the latter rests partly on the great accessibility of the zones. However, the method is superior to paper chromatography in that larger quantities of material can be handled, as well as volatile solutes. The method is chemically a gentle one, compared with some types of adsorption chromatography. Its use with high molecular weight substances is promising (see Chapter XIV).

Paper Partition Chromatography and Some Closely Related Methods

I. PRINCIPLE

The substances to be separated are distributed between two liquid phases, or a liquid and a gel phase. The liquid or gel stationary phase is supported on paper or treated paper or on a support of a similar nature (191). Paper chromatography differs from column chromatography in that there are no walls to retain the phases mechanically. Instead, the outer limits of the mobile phase are set by its surface tension.

II. CLASSIFICATION

Paper chromatography is so widely used that it is impossible to make more than a rough estimate of its application. The most complete and scholarly book on paper chromatography was published in 1954, and contained 3795 references (362). By 1956 the number could probably have been doubled. With the large number of people using paper chromatography, in different fields, and in the exigencies of different laboratory conditions, there has been a continuing healthy amount of gadgeteering of apparatus and variation of method. For this reason alone it is impossible to survey the field and describe all the variations of apparatus. Instead, what seem to be the basic requirements will be laid down and a few of the many satisfactory kinds of apparatus will be described and illustrated.

The same considerations apply to methods of operation. The variations that have been rung on procedure are extremely numerous, and no attempt will be made to review all of them. They will be used as sources of information about the fundamental principles that underlie paper chromatography. It is chiefly the principles that we are interested in.

In paper chromatography the mobile phase may flow over the stationary phase, driven in all cases by capillary forces, in a downward manner (aided by gravity). This is *downward flow* or *descending development*. The liquid may flow upward, against gravity. This is *ascending development*. It may flow horizontally. It may be made to flow radially outward from a central source of developer, "circular paper chroma-

tography," the *disk,* or *radial* technique. Combinations of these may be used.

The paper or other support may be in the form of strips, or sheets, or sheets rolled into a cylinder (213,359). It may be utilized as a rectangular stack made by folding a long strip backward and forward, and pressing the pleated strip into a solid pile (293,539); it may be utilized in the form of a stack of filter paper disks pressed together to make a column without walls, a *chromatopile* (247); or as a pack of long strips aligned one over the other like a deck of overlong cards, and clamped to make a rectangular column, a *chromatopack* (745). With any of these contrivances, the chromatography may be carried out by frontal analysis, development and elution analyses, gradient elution, displacement analysis, and carrier displacement analysis (Chapter II).

In every case the principles are the same. However, for the greatest explicitness, each of the important methods of operation will be illustrated and the less important ones called to attention in connection with related methods.

III. APPARATUS

The apparatus needed for ordinary paper chromatography is used in connection with the following steps in the analysis. These steps are common to almost all paper chromatography: the sample in solution is applied to the paper and dried on the paper. The paper is then placed in a chamber in an atmosphere saturated with the vapors of the developer. After the paper has become *conditioned*, or *accommodated* to the developer, development is begun. After the development the paper is removed from the chamber, dried, and examined for the zones, or spots of separated materials. This may have to be done by spraying the paper with a reagent, or dipping it in a reagent, that *reveals* the zones. In quantitative work, careful temperature control and attention to details are necessary, and in addition, some device for measuring the amount of material in the zone.

In this section only apparatus are described; methods are left till later.

1. Applicators

The mixed zone is formed on the paper usually from solution. There is therefore needed some way of placing a small drop, or narrow zone, of liquid on the paper at a predetermined place. The volume applied should preferably be known, as well as the concentration, though this is absolutely necessary only in quantitative work. The best devices to use are micro or ultramicro pipettes or burettes. These may be made (103,321,378,513,

568,622,790,938,953,959,1026) or purchased (see Appendix). They should deliver of the order of 1 to 25 μl. for ordinary work. Still smaller volumes may be necessary in some cases (844).

Where these instruments are not available and high accuracy is not required, very simple contrivances may be used: a toothpick, the end

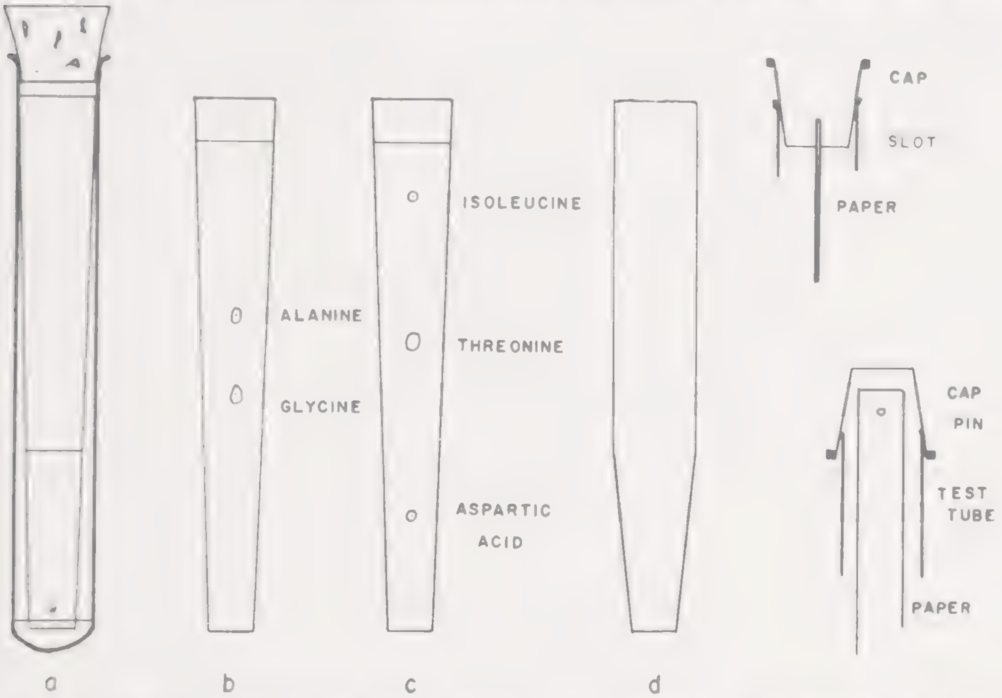


Fig. VII-1. Micro-method of paper strip chromatography. The sample is placed at the spot near the lower edge of the strip, which is then dipped into the developer in the test tube. The wide part of the paper at the top keeps the strip vertical. Beginning development is shown in (*a*), and two chromatograms in (*b*) and (*c*). (*d*). Strips may also be prepared from straight ribbons of paper (from a roll, for example) provided they are tapered part of the way so as not to touch the wall of the tube. After the developer has made its first rapid advance up the paper, contact of the paper with the wall seems not to be deleterious. As a convenient variation on this theme C. W. Partridge (708a) uses polyethylene stoppers that are slotted to hold the paper strip; or that are used as caps that fit over culture tubes. In this last case a pin is inserted through the cap to hold the paper strip. (Rockland and Dunn method (787).)

of which may be shaved down; a tiny bead on the end of a filament of glass rod; a fine capillary tube; a very small platinum loop on the end of a wire, fused to a soft-glass rod, of the kind bacteriologists use in making transfers (153). Devices that are disposable (as toothpicks, etc.) or easily cleaned (as a platinum wire or loop which may be ignited) are useful for quick qualitative work, especially when many solutions have to be applied, or "spotted."

2. Chromatographic Chambers

These can vary from simple to very complex. For **paper strips** perhaps the simplest is a test tube as is used by Rockland and Dunn (787). Test tubes with glass stoppers (Fig. VII-1) are best, but a cork or foil-covered cork will usually do to close the tube. For longer strips, cylinders such as are used for mounting biological specimens may serve, or graduated cylinders, if they can be closed tightly. For still wider strips, jars or crocks will serve provided they are glazed inside. If they are not glazed,

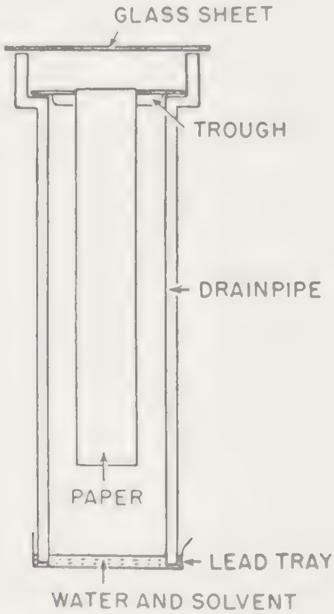


Fig. VII-2. Apparatus for paper strip chromatography. The paper strip hangs in a controlled atmosphere. The upper edge of the paper dips into the trough of developer liquid. (See Fig. VII-9.) (From Consden, Gordon, and Martin (191).)

a jar can be used for only one developer, because the liquid gets into the clay and cannot then be cleaned out. For long strips a drainpipe, such as Consden *et al.* (191) described can be used, or large-bore Pyrex piping which is now available (Fig. VII-2).

Sheets of paper can be handled in two ways. As extended sheets, they need a fairly large cabinet, which might be a metal box, or a glass aquarium (83), or one of the commercially available cabinets. The sheets can, however, be handled in jars or crocks by rolling them into a supported spiral (590), or into a hollow cylinder which is fastened by stapling (1001,1021), or lacing (433). (See Fig. VII-3.) Many ingenious variations of these arrangements have been published. For example, to run

a large number of sheets at once with the same developer, they can be spaced on a rack (115,214) and run in a tank or held horizontally on a special glass rack as in Fig. VII-8 and so on.

With **paper disks,** run in the radial method (an arrangement that is basically very old (793)), quite simple apparatus can be used. W. G. Brown (127) placed the filter paper between two pieces of glass, the upper of which had a small hole, $^1/_{16}$ to $^1/_8$ in. in diameter at the center. The filter paper and glass plate sandwich was held tightly together with

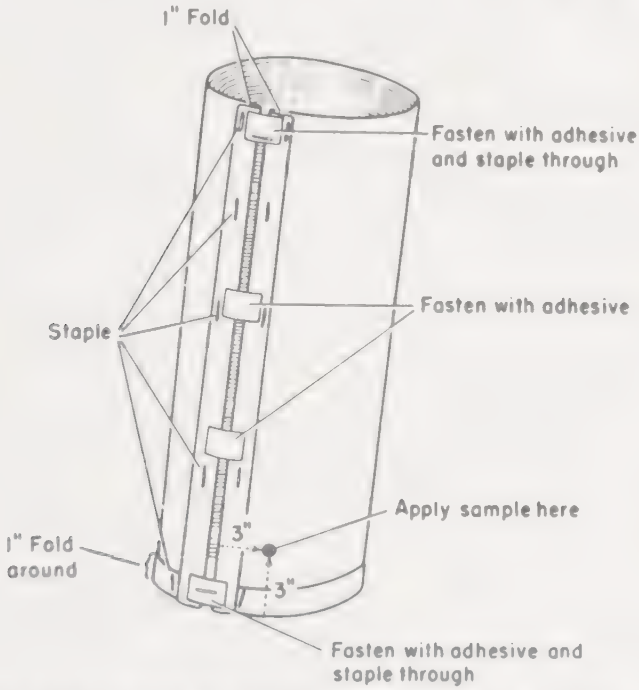


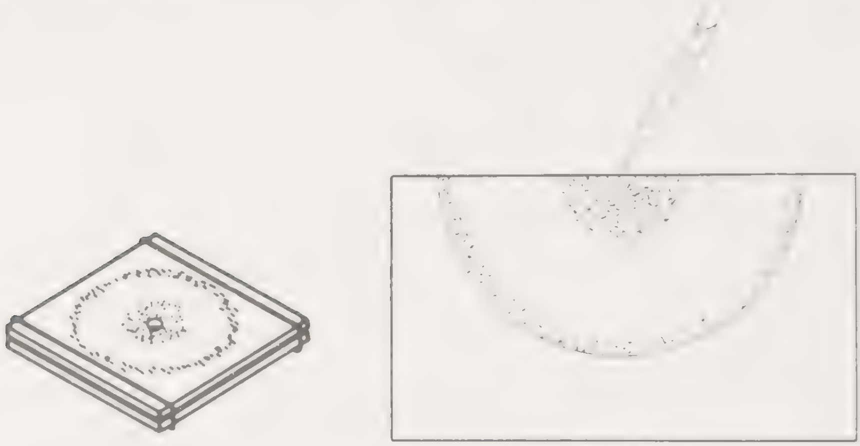
Fig. VII-3. The figure shows the method of folding and fastening large sheets of filter paper for upward-flow chromatography. The sample may be applied in the lower left-hand corner of the sheet as shown here, or in the lower right. The use of adhesive tape is seldom necessary.

rubber bands (Fig. VII-4). The mixture is introduced through the hole, followed by developer, dropwise. The plates serve to suppress evaporation. The method is rapid and elegant. It requires the use of smooth paper; creped paper is unsatisfactory. (The hole in the glass plate may be bored with an off-center drill (a broken drill) lubricated with turpentine.)

A still simpler arrangement was suggested by Rutter (797). The filter paper is supported on the top of a shallow dish, and the developer is fed to it through a wick. Two parallel cuts are made from the periphery of the disk to a line through the center of the disk perpendicular to the cuts, so that the cuts are equally spaced from the center of the disk. This

produces a flap of paper. The flap is now bent at right angles to form the wick and is cut off to the right length so that it reaches the bottom of the dish (see Fig. VII-5). The rate of development with a given paper is controlled by the width of the wick.

The chamber for such a setup is conveniently made from two larger Petri, or crystallizing, dishes (750,751) (Fig. VII-6). The disk should best lie flat, and not be crumpled or folded. Various alternate ways of



(a) (b)

Fig. VII-4. Chromatography with a filter paper disk. (*a*) The sheet of filter paper (or a layer of adsorbent) is placed between glass plates to suppress evaporation of solvent. The substance to be chromatographed is introduced through the hole in the center of the upper plate, and followed with developer. (*b*) A piece of filter paper, held between two glass plates, with the upper edge straight and flush with the glass, can be used for quick examination of a substance or mixture. Developer is applied slowly with a capillary pipette. It is easy to overload these systems.

feeding the developer to the chromatogram have been devised: a wick of rolled filter paper, inserted through a hole at the center of the disk (750) and cut at the lower end to form a brush, or a small cone of paper, with its apex at the center of the disk (65), or a wick of cotton thread (433,839) or a self-regulating pipette (117). Of all the methods, that using the self-wick cut from the disk is simplest, while in our experience that method using a cotton wick (white darning cotton), threaded through the center of the disk, with a small knot to keep it from pulling through, seems to give best results. Many other devices may be used with satisfaction.

In work with light-sensitive substances the cabinets should, of course, be opaque or the analysis carried out in a darkened room (872). Red-glass jars may be used (124). With oxygen-sensitive substances, the cabinets

are kept filled with inert gas, which should be bubbled through developer (or the aqueous equilibrated phase, see below) before being led into the cabinets, so as not to disturb the concentration of the vapor atmosphere.

3. Supports for Papers and Troughs

In upward development methods (which are the simpler kinds with paper strips and sheets) the paper dips into the developer. Strips may be

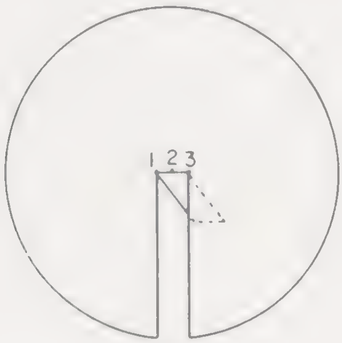


Fig. VII-5. Radial chromatography. A filter paper disk is cut as shown. The initial spot of mixture may be placed on the wick, just below where it is bent, or at the bend, the center of the disk. Two or more mixtures may be compared by placing spots on an arc at the bend of the wick, as shown at 1, 2, 3 (894).

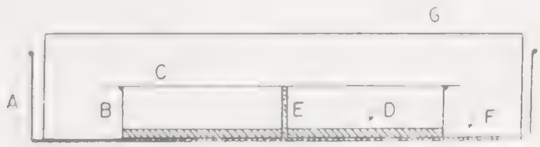


Fig. VII-6. Chamber setup for radial chromatography. *A*, Inverted 14 cm. Petri lid; *B*, 9 cm. Petri dish; *C*, filter-paper disk; *D*, mobile phase; *E*, filter paper wick; *F*, filter paper saturated with water, or aqueous phase; *G*, inverted bottom of larger Petri dish. (After Proom and Woiwod (750).)

hung from a hook or small clamp fastened to the stopper or lid of the chamber, or as in the Rockland and Dunn (787) method, they may be tapered and hung by friction against the wall of the tube at the top part of the strip. Sheets may be formed into a cylinder (435,1021) or a spaced spiral (590), and stood up in the developer, as illustrated in Fig. VII-7.

In the downward development method the sheets or strips may be hung over the edge of the trough used to contain developer, as in the original

design of Consden, Gordon, and Martin (191). Here the trough was supported on the shoulder of the drain pipe that was used as a chamber (Fig. VII-2). Many other devices have been described: a crock with a support for paper sheets or strips (531), an aquarium with supports fastened in with paraffin (83) or containing a rack to hold the sheets (115,214). Various kinds of racks to hold the troughs have been illustrated by Hais (362). A friction support that fits inside a cylinder has been designed by Porter (746). A rack has been devised for use in a shallow chamber (625) (Fig. VII-8), and so on. The devices for feeding disks have already been described (Fig. VII-6).

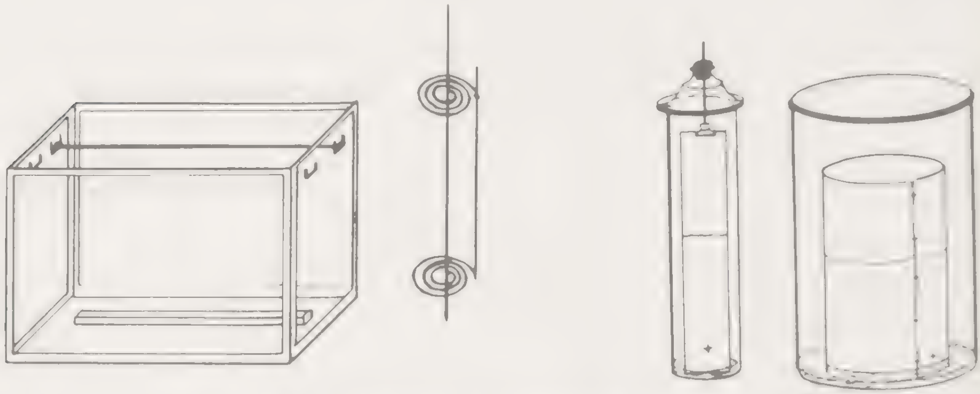


Fig. VII-7. Methods of supporting the paper.

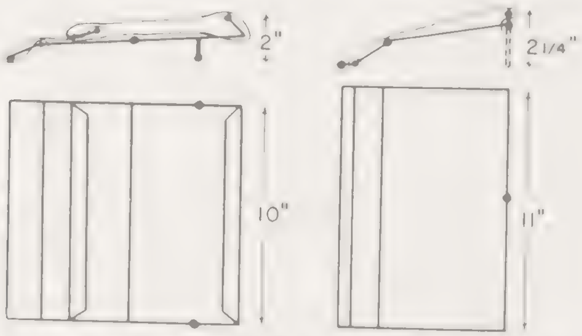


Fig VII-8. Rack for a shallow chamber. The rack is of glass. The lower edge of the paper dips into the developer. (After Meredith and Sammons (625).)

4. Troughs

Troughs for holding developer are not needed in the upward development method, or with disks of paper. In the downward method they are usually needed, and may consist of beakers or Petri dishes, or, for wider strips or sheets, glass or stainless steel troughs, shown in cross section in Fig. VII-9. These may be made of glass as described by Longen-

ecker (587), or they may be formed from stainless sheet by welding. However, both glass and stainless troughs can be purchased, as well as all apparatus for paper chromatography (see Appendix).

What has seemed to the author to be the most controllable arrangement for paper chromatography is that described by Irreverre and Martin (433) (Fig. VII-10). With this apparatus, as with few of the others, the rate of development can be very precisely controlled. Such control can be

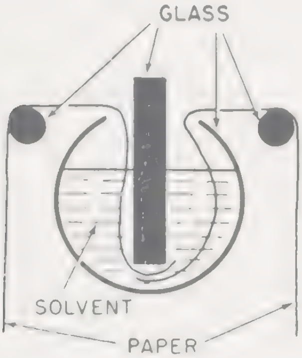


Fig. VII-9. Cross section of a trough in use. One end of the paper strip or sheet dips into the developer solvent and is held down by a plate. Glass spacer rods hold the paper away from the edges of the trough to prevent siphoning of the liquid. The initial spots of mixture are placed along the paper just below the spacer rods. The trough may also be made of metal.

obtained in other ways, as by changing the width or thickness of the wick in radial chromatography, or by using larger or smaller, thinner or thicker, strips of paper, sewed or laced on, as feeders to the main strip in upward or downward development.

Sometimes it is necessary to continue development for a long period, longer than it takes for liquid to reach the end of the paper. In such cases (as with very low R_F substances) a wad of cotton or paper may be fastened (stapled, or sewn) to the end of the paper to absorb the developer (365), or, in downward development, the lower edge can be cut in a zigzag to form points, from which the liquid will drip (405,452). Fischbach and Levine (288) extend the upper edge of the paper (upward development) through a slot in the lid of the chamber or through a slot in the cap of the cylinder and allow the solvent to evaporate either without further aid or in a draft (a hood). This permits using jars or cabinets that are no deeper than the expected length of the chromatogram, and is reported to give less diffuse zones and better separations than a continuous descending method.

5. Dryers

After development, excess developer is removed by drying the chromatogram in a hood either without applied heat or with the use of an infrared lamp or electric heater with a fan (a hair-dryer or a room-warmer). There are available safety drying ovens, with forced ventilation, and protection against explosive mixtures of vapors.

6. Sprayers

In order to reveal zones or spots of colorless substances on paper chromatograms it is common practice to use color reagents. For example,

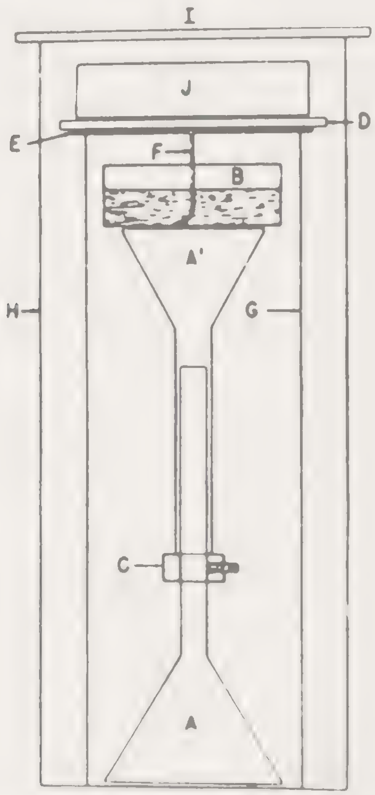


Fig VII-10. Apparatus for close control of paper chromatography. *A*, 115-mm funnel; *A'*, 80-mm. funnel. *B*, crystallizing dish containing mobile phase. *C*, Teflon ring, or rubber ring held by friction, and supporting the stem of funnel *A'*. A glass-tube sleeve may be used to support funnel *A'* if its stem is not large enough to go over that of funnel *A*. *D*, glass plate. *E*, filter-paper disk. *F*, wick of cotton thread. *G*, filter-paper cylinder. *H*, glass cylinder. *I*, plate glass cover. *J*, additional weight on top of plate glass *D*. The speed of development can be accurately controlled by the number of strands of wick used. The development can be terminated at any point by controlling the amount of liquid in *B*. (After Irreverre and Martin (433).)

ninhydrin may be used for amino acids and related compounds. These reagents may be applied either by *dipping* the sheet, from which developer has been removed, in a solution of the reagent, and letting the excess drain off, or by *spraying* or *brushing* the reagent onto the sheet. A number of designs for atomizers, or sprayers, have been published (1011). Metal and glass atomizers can be purchased. The all-glass Nebulizer, which can be obtained cheaply, gives a very fine-particle mist which does not, however, deliver much liquid per square centimeter sprayed, and so has to be passed over the chromatogram many times.

After the reagent has been applied it may be necessary to heat the chromatogram, in which case an oven of the type described can be used.

Other methods of applying reagents to detect zones will be described in connection with recognition of zones.

IV. PAPER AND OTHER SUPPORTS

The paper in paper partition chromatography should function solely as a support for the stationary phase. This implies that it should interact in a sorbtive manner with the molecules of the phase, but should not take up any of the components of the mixture being separated. Since the paper is a hydrophilic substance (though it is wetted by all solvents), it tends to sorb the more polar components of any binary mixture, and so it is that normally the stationary phase will be the more polar of the two, the mobile phase being less polar. In *reversed phase chromatography* (89) the paper or other support is treated to make it lipophilic, whereupon the stationary phase becomes the less polar one.

There are a number of properties of paper that are of interest to the chromatographer *because they may affect* R_F (170,362,831,1040). They may be classified as bulk properties and as molecular properties. Filter paper is a mat of interlaced and to some extent bonded cellulose fibers.

1. Bulk Properties and Molecular Level Properties

The chief bulk properties of interest to the chromatographer are the purity, the porosity, and the strength of the paper.

There are a very great number of kinds of filter paper available, but relatively few are used by chromatographers. These are the purified analytical-grade papers, as distinct from industrial papers that are brown, or gray, or highly creped, and as distinct from special papers that are not readily available. There is also, of course, a tendency to follow what other workers have found to be successful practice, and this has tended very much to limit the number of kinds of papers in use. Too little is known in any case about what makes good chromatographic paper.

Since all good filter paper is made of highly purified cellulose, the chief differences between kinds of paper, at the bulk level, result from the mechanical treatment, during the paper-making process, that separates and breaks fibers, mats them, and in the process controls surface area and porosity. These features of paper have been discussed to some extent by Balston and Talbot (27).

The differences at the molecular level also result from this treatment and comprise (apart from the incorporation of impurities) differences in the arrangement of cellulose chains, that is, differences in ratio of oriented to nonoriented portions of fibers. The cellulose fibers seem not to be uniformly constructed but to consist of regions where there is a regular, repeated alignment of chains of glucose residues, the crystalline regions, and alternating with these regions in which the chains are more or less randomly linked and unaligned, the amorphous regions. According to Hermans (386), the crystalline portions are fairly impenetrable to liquids. It is in the amorphous regions that imbibition leading to swelling may occur. *Swelling* of this kind is accompanied by dimensional changes in the fibers (460). In *porous imbibition*, the liquid is taken up by capillary action in pores already present in the paper, and the dimensions of the paper do not necessarily change.

Both kinds of imbibition seem to be produced by polar liquids. Kress and Bialkowsky (460) have ranged liquids in order of swelling ability thus: formamide $>>$ water, slightly greater than ethylene glycol $>$ methyl alcohol $>$ furfuryl alcohol $>$ ethyl alcohol $>$ furfural, slightly greater than propyl alcohol, slightly greater than *n*-butyl alcohol, slightly greater than fuel oil (a mixture of hydrocarbons slightly heavier than kerosene). They point out that liquids with hydroxyl or potential hydroxyl groups and high dielectric constants show a high rate of swelling of cellulose, whereas liquids of low dielectric constant show little ability to swell the fibers. Within certain concentration ranges aqueous acids and bases swell cellulose, and produce quite complicated phenomena upon which a great deal of work has been done (393,702,802).

All mobile organic liquids and most inorganic ones rise into filter paper strips that are dipped into them. The phenomenon here is porous imbibition, and it occurs with liquids such as hydrocarbons that do not swell cellulose appreciably. Swelling imbibition is usually a slower process than porous imbibition.

At the molecular level also, there are probably some differences among papers in the availability of hydroxyl groups. The capillary rise of polar liquids would be expected to be mediated by polar interactions of the kinds discussed in Chapter III between the liquids and the paper fiber surfaces (crystalline as well as amorphous), leading to mutual lowering

of surface tensions (low interfacial tension). The capillary rise of non-polar liquids may be facilitated by the mutual interaction through hydrogen bridging, or other interactions of neighboring OH groups in the fiber surface, thus leading to a depolarization of the fiber surface. Philippoff has pointed out that because paper contains water the rise of a liquid may involve spreading of the organic phase on an effectively aqueous surface (6).

At the molecular level, too, account must be taken of the presence of aldehyde or potential aldehyde groups, carboxylic groups that can act as exchangers, and salts of various kinds, present in traces, either left by the manufacturing process or built into the fiber by the plant during laying down of the cellulose fiber.

Balston and Talbot give the following information (27). "As a general indication of the purity of the cellulose, Whatman papers commonly used for chromatography have an α-cellulose content of 98–99%, β-cellulose of 0.3–1.0% and pentosans in the region of 0.4–0.8%, calculated on a moisture-free basis. The ether soluble matter varies between 0.015–0.05%, ammonia 0.001–0.006% and organic nitrogen 0.001–0.01%. These figures of course vary slightly from grade to grade depending on the raw materials used and the treatment received. The mineral content expressed as an ash varies from 0.07% in the case of qualitative papers to 0.01% in the case of double acid-washed papers."

2. Porosity

A tremendous amount of study has been given to porosity phenomena in paper (170,702). If a strip of filter paper (or any kind of porous paper) is dipped into a liquid that wets it, the liquid rises into the paper. The first rise seems to be quite turbulent, in porous papers (505), and is then followed by a smoother and progressively slower rise as the front of the rising liquid draws away from the surface of the bulk liquid into which the paper dips. In a closed container the rise of liquid can continue for a long time. For example, Wood and Strain (1022) found that the rate of rise of water into Eaton-Dikeman paper, Grade 301, was 0.1 mm./min. at the end of about 24 hr.; 0.09 mm./min. at the end of about 48 hr.; and after three weeks the liquid was still rising, but had slowed down to less than 0.01 mm./min. (In a packed sand column in a tube, a paraffin oil was found to be still moving after one year, the height then attained being some two to three times the height risen in the first 24 hr. (356).)

Normally, it is only the first relatively rapid rise that is of importance for chromatographers. If paper chromatograms are to be run for a long time, as in continued development, this is usually done by the downward method.

Downward flow starts out initially at about the same rate as upward, but it decreases only slowly with time. The same is true of radial flow: in one case the rate in radial flow decreased from 0.50 to 0.28 mm./min. in 95 min. (1022). It is sometimes observed that filtration rate of liquids through filter paper and glass, porcelain, or quartz filters slows down with time (87,857), even when the paper is folded in a funnel and pure water flows through it.

There is a bulk property of papers that has an effect on velocity of flow of liquid. The liquid flows faster along the machine direction than across it. The machine direction is the direction parallel to the forward movement of the paper in the paper-making machine. The two directions can be distinguished by various tests which have been summarized as follows (170):

"1. Ease of bending. A paper strip cut in the machine direction tends to bend less easily than one cut in the cross direction.

"2. Tendency to curl. If pieces of paper are floated on water, or, if very absorbent, are exposed to water for a few seconds, the paper tends to curl, with the axis of curl parallel to the machine direction.

"3. Capillarity. Water usually rises faster up a strip cut in the machine direction. A drop of water placed on a sheet may spread to an oval, with the longer axis parallel to the machine direction. (There are some commercial papers in which machine direction cannot be determined by flow of solvent.)"

4. Behavior to tearing. In general, papers tear in long straight strips parallel with the machine direction, but across the machine direction the tear is irregular.

These behaviors are consistent with a preferential lining up of the cellulose fibers in the *machine direction* as the paper is made. This orientation may be increased for special purposes. Calculations of average pore diameter of a number of papers (not filter papers) indicated a larger diameter in the machine than in the cross direction (296).

There is a correlation between pore diameter (porosity) and rate of rise of liquid into porous substances. Within limits, the larger the pores the faster the rate of rise.

Another bulk property of importance is the *homogeneity of the paper*. All strips and sheets should be held in front of a bright light, and inspected for imperfections such as thick or thin places, holes, opaque inclusions. Only homogeneous strips or sheets can be used in good work.

The rates of rise of fluids in paper are conveniently compared in terms of dh/dt at a given height of rise h. This can be obtained by timing the rising front of liquid as it reaches increasing heights from the surface of the liquid or from a starting line near the surface, and applying the relation of Müller and Clegg (669):

$$h^2 = Dt - b \tag{1}$$

where h is the height risen (in centimeters) in the time t (in minutes) and D and b are constants. From this relation $dh/dt = D/2h$, and D can readily be calculated from the data using the slope formula and equation (1). In an investigation of the rate of rise of 141 liquids into Whatman No. 1 paper (all in the machine direction), three regularities were found (6):

"With a given group of homologs the rate of rise decreases rapidly as the number of carbon atoms is increased, with the exception (commonly found among other properties of homologs) that the first few members of the series with one to three carbon atoms do not fall in line. This is evident with the normal acids, 1-bromides, ethers, ethyl esters, and methyl ketones.

"The overall pattern obtained in comparing dh/dt at 6 cm. with number of carbon atoms in the molecule indicates that for corresponding compounds the less polar the substance the more rapid will be its rate of rise.

"The corresponding compounds (or series of homologs) fall in the general order: hydrocarbons, faster than ethers, faster than esters, halides and ketones, faster than acids and alcohols."

The factors governing the penetration of paper by liquids have been analyzed by numerous investigators. Peek and McLean (719) derived an equation to fit the data:

$$\frac{dh}{dt} = \frac{A\gamma}{4\eta h} - \frac{Bdg}{8\eta} \tag{2}$$

in which γ is the surface tension of the liquid (which is assumed to wet the paper completely), η is the viscosity, d the density, g the acceleration due to gravity, and A and B are constants which are dependent on the pore-size distribution in the paper; h and t have the meaning given in equation (1). Wood and Strain (1022) felt that in addition to the parameters in equation (2) there might be needed a term to represent the force due to a diffusion potential of the liquid along the fibers of the paper. An examination of the rate of rise of many liquids into a filter paper by Ackermann and Cassidy (6,492) led them to conclude that the overriding factor in rate of rise is the viscosity of the medium (η, in the equation of Peek and McLean) and neither density nor surface tension by themselves. They concluded also that for ordinary chromatographic practice the easiest way to determine rate of rise is to try out the system with the paper in question, using the apparatus of Rockland and Dunn (787).

These considerations are important because in general, but not always in comparing *different papers*, the faster ones give higher R_F values (433,492,

1040). With the *same paper*, in which the rate of flow of developer is controlled there is little effect of flow rate on R_F for small differences in rate (495), but when the flow is considerably slowed the R_F was slightly but measurably decreased (433).

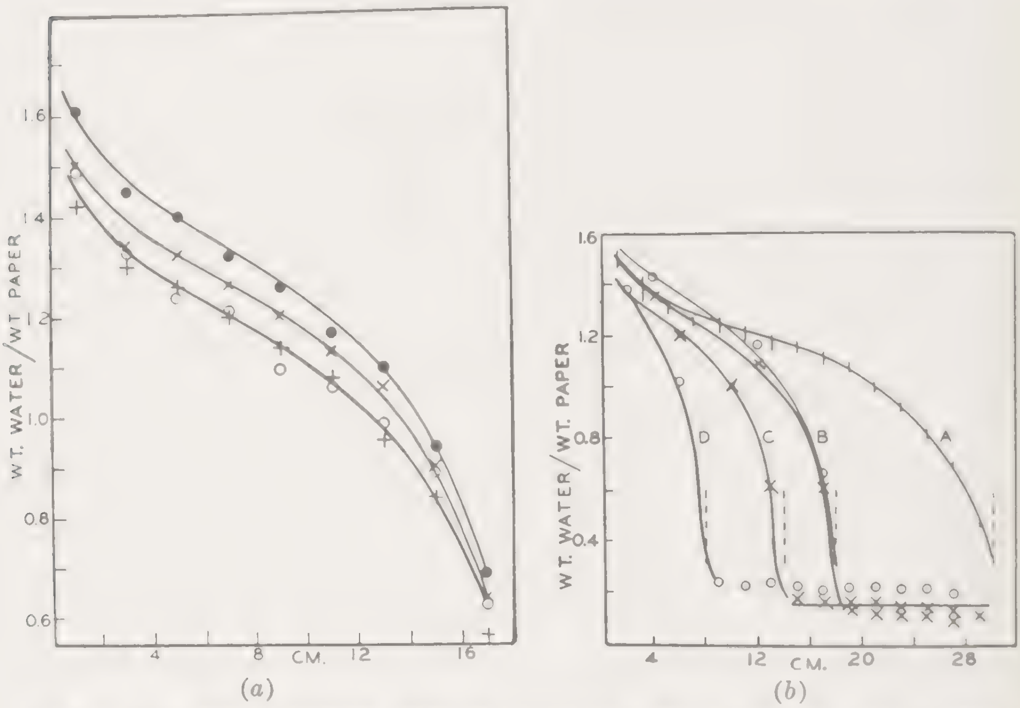


Fig. VII-11. Effect of various conditions, including distance run, on mass gradient in papers. (*a*) Mass gradients obtained under various conditions. (× Upward development at 25.1°C., average of three runs. ● Upward development at 4°C., average of three runs. + Upward development of paper cut 90° to machine direction, at 25.1°C., average of two runs. ○ Downward development at 25.1°C., one run. Solid lines drawn to aid visualization.) (*b*) Effect of distance moved by front on mass gradient profile. (*A*, Average of two runs, conditioned and developed at 25.1°C., and run to 30 cm. above water surface. *B*, Developed to 18 cm., *C*, developed to 11, *D* developed to 8 cm. above water surface.) Sectioning and analysis were continued above front as shown by nearly horizontal points from front on, and these points represent the ratio of weight of water to weight of dry paper for conditioned paper (as at foot of (*b*)). (× Strips conditioned and run at 25.1°C. ○ At 4°C.) All values are slightly low because of evaporation during sectioning. All papers Whatman No. 1. (From Ackerman and Cassidy (6).)

A further phenomenon of importance to the later discussion of the effects of various factors on the R_F value is the *distribution of liquid* along the paper. Krulla (505) showed that there is a mass gradient in the fluid as it rises in filter paper. These data have been fitted with an equation by Fujita (304), and the subject has also been investigated lately in its implications

for chromatography. Wood and Strain (1022) confirmed that the amount of liquid in the paper per unit area decreased upward to the front in upward rise of the liquid in Eaton-Dikeman Grade 301; with downward flow the distribution was fairly uniform, and with radial flow there was a marked gradient. Profiles of the gradients found by Ackerman and Cassidy with Whatman No. 1 paper are shown in Fig. VII-11. Somewhat similar profiles were found with 10 other papers (6).

Evidently, the downward, upward, and radial methods differ, from the bulk-phenomenon point of view at least, in the following ways. In both the downward and upward there is a similar mass gradient of solution: a heavier amount of liquid in the paper at the surface of the solution where a meniscus is present, then a more constant amount for some distance (if the front is far enough away (Fig. VII-11*b*, curve *A*), and finally a rapid drop in amount of liquid to the front, where again the low level of liquid becomes constant and represents the amount in the paper in the presence of a saturated atmosphere. While the mass gradient profiles are essentially the same, the velocity of flow in downward development remains essentially constant (with water), according to Wood and Strain (1022), though Kowkabany (491) found considerable slowing down when collidine or phenol developers were used. In upward development the velocity of flow decreases quite rapidly as the front draws away from the reservoir of developer. In the radial flow method, the velocity of flow of liquid decreases, but not so rapidly as in upward development; and the mass gradient becomes very pronounced as the area to be covered by the liquid continually increases with radial distance traveled by the front. The meaning of these phenomena in terms of R_F will be discussed fully below.

3. Purity and Purification

Most papers used in chromatographic work are quite pure, and some especially purified ones are now available. However, such minute amounts of materials can be handled chromatographically on paper that even most minute amounts of impurities can cause difficulties either by masking zones, or by distorting them, or by aiding catalytic changes in the components, or by producing artifacts. These phenomena are discussed in detail among the factors that affect the sharpness of zones. Here some information is given about purifying paper.

To remove the effects of heavy metal salts, these may be complexed on the paper by adding to the developer or the atmosphere of the chamber suitable reagents (such as H_2S, HCN, NH_3, $K_4Fe(CN)_6$, α-benzoinoxime) Or the paper can be washed with dilute nitric acid (1*M*) (1022); or 0.01*N* hydrochloric acid (27); aqueous 8-hydroxyquinoline (27); or 0.2% Versene (ethylene diamine tetraacetic acid) neutralized to pH 8.5 with

dilute NaOH (259); or unneutralized; and so on. In any case the washing should be done countercurrently, by downward development, for greatest efficiency. Hanes and Isherwood (365) have described a method for washing a large number of sheets. A large "Büchner" of methacrylate polymer was made to take 30 to 60 sheets at once, and these were washed with 2*N* acetic acid.

In pure water, filter paper takes on a negative *charge* implying adsorption of OH-ions (the isoelectric pH of cellulose is 2.6) (313), and when a strip of paper is dipped into solutions of negatively charged colloids they may rise with the front, while positively charged colloids may be flocculated. This behavior has been extensively studied and appears to be not without exceptions. The operative factors seem to be chiefly salt concentration in the solution and concentration of colloid, as well as charge on the colloid. The phenomenon is of interest in connection with deposits sometimes left at the place of application of the initial mixed zone. Even differently charged bacteria may be separated by rise into paper (301).

4. Modified Filter Papers

Papers may be modified in two quite different ways, by fillers of various kinds, such as adsorbents, absorbents, or buffers; and by chemical modification of the surface structure of the paper.

A. IMPREGNATED PAPERS

Papers are impregnated for reasons which fall into two chief categories: either to guarantee the maintenance of a desired pH; or to produce reversed phase chromatograms. There are other objectives, of course, and papers impregnated with alumina or other adsorbents have been used. Papers filled with diatomaceous earth, or charcoal, are commercially available. These latter applications belong in the chapter on adsorption chromatography, and are treated there (Chapter VIII, Section V).

Buffered Papers. In work with amino acids and some other amphoteric substances it has been found that pH control is important for ensuring reproducible R_F values and well-formed zones (375,554,581). It is not satisfactory to buffer the mobile phase alone (581). The stationary must also be buffered. The ionic strength of the buffer seemed to have little effect on the R_F of amino acids. McFarren (581) also found that buffers of 0.067, 0.09, and 0.143 molarity did not cause changes in R_F values. However, the nature of the buffer salts is important. Thus a phosphate buffered alcoholic developer would be expected to yield different results from a borate buffer, especially too with phenolic substances. Alcohols and phenols interact with borate

The usual procedure (581) is to dip the paper in the approximately 0.066*M* aqueous buffer solution of the desired pH, hang it by one edge, and let it air-dry. The organic phase is equilibrated against a fresh lot of the same buffer solution by shaking in a separatory funnel. When the layers have separated, the buffer layer is used in the chamber to condition the atmosphere (581).

Paper has been impregnated with many substances other than buffers (478). Sporer, Freed, and Sancier (872) impregnated paper with sugar, and used it for the separation of chlorophylls. The papers were soaked in an aqueous solution of 0.18 g. sugar per milliliter solution. The sugar used was Jack Frost 6x, and the solution was filtered through a Büchner before use. The papers were then dried at 100°C. The developer was 0.5% *n*-propyl alcohol in *n*-hexane. The chromatography, including application of the initial zone to the paper, was carried out under nitrogen, in a semi-darkened room.

Zaffaroni and co-workers (1027) and many others (84,362,529) have reported on papers impregnated with stationary phases of a polar nature (such as formamide or propylene glycol).

B. CHEMICALLY MODIFIED PAPERS

Some commercial papers have been treated with a polymer (such as melamine) to confer increased wet strength. Such papers would show different R_F values for acids and bases than their untreated counterparts. Paper may be superficially acetylated (or acylated). The procedure of Kostir and Slavik (489) is to acetylate the paper in a closed vessel at 38°C. for 20 hr. The acetylation solution is made by shaking equal volumes of pure toluene and acetic anhydride with 0.1 volume of concentrated sulfuric acid, letting settle, and discarding the sulfuric layer. The solution is stirred occasionally during the acetylation. After 20 hr., the paper is drained, air-dried for 9 hr., and then washed with methanol. Such paper was used for separating dinitrophenyl hydrazones. It held a stationary phase of *n*-octanol fairly well in the presence of a mobile phase of benzene: petroleum ether (1:1, v/v); or halogenated hydrocarbons such as chloroform, trichloroethylene, chlorobenzene, in the presence of a mobile phase of 80% ethyl alcohol or 80% isopropyl alcohol, but the system seemed of somewhat uncertain stability.

It is somewhat difficult to acetylate paper without destroying its structure. The subject has been reviewed by Buras and Hobart (142). Davis, McMahon, and Kalnitsky were unable to effect satisfactory separations of sterols using acylated filter papers (218).

Conversion of the paper surface to a lipophilic state for the purpose of *reversed phase* chromatography may be done by partially filling

the fiber structure, or coating it, with lipophilic substances or by chemically modifying the surface. Boldingh (89) immersed paper in a dilute vulcanized rubber latex solution. The strips ended with 30% of their weight of rubber. They were air-dried, rinsed with alcohol and acetone, and stored under acetone. Such strips were used to separate higher aliphatic esters with a benzene stationary phase and methanolic mobile phase. Other workers (122,1015) have coated paper with vaseline. Winteringham *et al.* (1014,1015) passed the paper through 2.5% or 3% (w/v) USP petroleum jelly in ether. This gave a petroleum-impregnated paper which was used with an ethanol : water : ammonia (sp. g. 0.90) (80:15:5 by volume) mobile phase to separate bromine derivatives of DDT and other substances. Paper has also been treated with silicone to produce a lipophilic surface. T. H. Kritchevsky and Tiselius (592) passed strips of Munktells 20,150G filter paper through a 5% (by volume) solution of Dow Corning Silicone No. 1107 dissolved in cyclohexane. The papers were blotted, then dried at 110°C. for 1 hr. They were used to separate steroids, using a mobile phase made by mixing water, absolute ethanol, and reagent chloroform in the ratio 6:10:10 by volume. This mixture was let stand 1 hr., even though it separated promptly. The lower, nonpolar phase was used in the chamber to condition the paper (1 hr.) and the upper, more polar phase was used as the mobile phase.

Chemical modification of paper to produce a lipophilic surface was done by D. Kritchevsky and Calvin (500). They treated the paper with Quilon, stearato chromic chloride. This allowed them to separate steroids, with an alcohol, or alcoholic mobile phase. The paper is impregnated either by spraying on a solution that is 2% in Quilon and 2% in "neutralizer" (duPont Product Information Bulletin, "Quilon," January, 1950) and drying at 100° to 110°C.; or by dipping in the solution, draining, and drying (501). Kritchevsky and Kirk report that the paper need only be thoroughly wetted with the reagent mixture and that a drying time of 5 to 10 min. is usually adequate and need not be prolonged. The paper so produced is not wetted if the alcoholic mobile phase contains too much water. With aqueous methanol the upper limit of water content is 20%. These authors also tested other chromic chloride complexes as modifying agents for the paper. Of these gluconochromic chloride seemed best, but gave more variable R_F values for several sterols and steroids than the Quilon paper.

5. Choice of Paper

So little is known about the properties of paper in relation to their rational chromatographic use that except for some fairly obvious considerations (such as that thick papers will usually hold more fluid than thin)

that very fast papers may give poorly defined spots; and that melamine-treated paper will retard acid zones) the choice of paper usually rests in the end on imitation of the successes of others, or on trial. A great deal could be done in this area to smooth the lot of the chromatographer. Kowkabany and Cassidy (491,494) made a study of 75 filter papers with a view to finding regularities in behavior that might aid the practicing chromatographer. Papers of the Eaton-Dikeman (E.-D.), Reeve Angel (R.A.), Schleicher and Schuell (S.S.), and Whatman (W.) brands were examined. Most of the papers were tested with five developers, and with special mixtures of amino acids designed to include acidic, basic, aliphatic, and aromatic acids, with some R_F values close together.

TABLE VII-1
Approximate Speeds of Some Filter Papers (491)

Developer Filter paper[a]	Collidine	Phenol	*n*-Butyl alcohol–ammonia	Sec. butyl alcohol–formic acid	Sec. butyl alcohol–ammonia
W. 1, 2, 3	I	I	I	I	I
W. 4	I	I	I	F	I
W. 5	S	S	S	I	S
R.A. 201	I	I	I	I	S
204	S	S	I	I	S
211	I	I	I	I	I
S.S. 507	S	S	S	S	S
589 Black R.	F	F	F	F	F
589 White R.	I	I	F	I	I
589 Blue R.	S	S	I	I	S
598	F	F	F	F	F
E.-D. 4 Purity	I	I			
5 Purity	I	I			
609	—	F			
611	S	I			

[a] W., Whatman; R.A., Reeve Angel; S.S., Schleicher and Schuell; E.-D., Eaton-Dikeman. F = Fast, 6–8 hr. to move 40 cm., I = intermediate, 13–25 hr. to move 40 cm., S = slow, 35–60 hr. to move 40 cm. In all cases flow was downward.

The results are shown in Table VII-1. The Eaton-Dikeman papers were not available at the time the tests in the last three solvents could be made.

The findings are subjective. They may be supplemented by the following observations, based on the same criteria and systems (491). Papers with intermediate rates of flow, e.g., requiring 15 to 25 hr. to move 40 cm. in a downward direction, seem best for qualitative analysis in phenol, collidine, and secondary butyl alcohol-formic acid developers. Fast papers (6 to 8 hr. for 40 cm.) seemed preferable for *n*-butyl alcohol runs. Slow papers

seemed best (35 to 60 hr. for 40 cm.) for secondary butyl alcohol-ammonia. A slow paper such as S.S.507 is useful for quantitative work because of the general sharpness of the zones and the lack of fast-running "beards." The variations in R_F values for the same amino acid found with different papers,

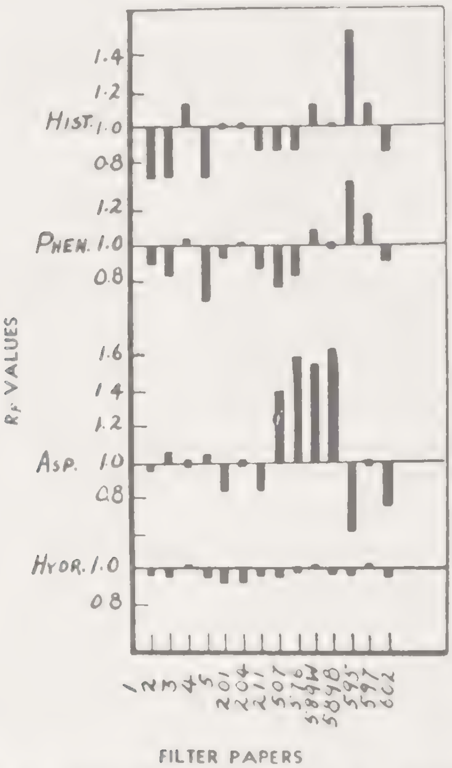


Fig. VII-12. Relative R_F values of amino acids on different papers. R_F values for four amino acids, two each in two different solvents, are shown for fifteen filter papers. In each group the papers were run at the same time in the same cabinet, and the same mixture of amino acids was used with each paper. The R_F values are plotted on the ordinate relative to Whatman No. 1 paper, which is taken as the norm. Hist., histidine; Phen., phenylalanine, both in 1-butanol saturated with 3% aqueous ammonia. Asp., aspartic acid; Hydr., hydroxyproline in phenol 80%, water 20% by weight. On the abscissa are listed the filter papers: W refers to White Ribbon; B to Blue Ribbon. The first five are Whatman papers, the next three are Reeve Angel; the rest are Schleicher and Schuell papers. (Eaton-Dikeman, Munktell and other papers were not available when this comparison could be made.) (From Kowkabany and Cassidy (494).)

and shown in Fig. VII-12, may be useful in specific separations. Fast papers such as S.S.604 and 598 can have advantages where speed of analysis is a primary factor.

Rockland, Blatt, and Dunn (785) have reported the examination of 13 papers on the basis of 7 characteristics. This allowed them to give the

papers the ratings shown in Table VII-2. Some additional properties of filter papers are shown in Table VII-3.

Some papers that have been recommended for chromatographic use, by the manufacturers or by various investigators, are listed with sources and characteristics in Table VII-4. It should be noted that some papers can be bought in rolls.

6. Supports Other than Paper

There is becoming available "paper" made from a number of substances other than cellulose, namely, nylon, a polyamide fiber; Orlon, an acrylic fiber; Dacron, a polyester fiber; cellulose acetate; Teflon, polytetrafluoroethylene; glass; quartz; 95% glass, 5% rope; 15% asbestos, 85% esparto, cotton, and manilla waterproofed with silicone; and probably others. They are so new that not much is known about them except that some have poor wet-strength characteristics.

Klingsberg (481) has reported that sharp separations of vat dyes in their leuco forms have been obtained by chromatography on Dacron fabric, and that nylon and Orlon have also been found suitable for these separations.

V. MOBILE PHASE. DEVELOPERS

The primary function of the mobile phase is to move the zone, that of the stationary phase is to retard it; and that of the system composed of the two is to differentiate, in their relative effectivenesses, between the components of the mixture to be separated. The choice of phases will be dealt with in a later chapter (Chapter XIV), and the relationship of the phases to R_F will be discussed in a later section of this chapter. The term *developer* is sometimes replaced with *solvent*, and sometimes with *eluent*. A discussion of the meanings and uses of these terms is given in Chapter VIII, Section VII.

In partition chromatography a binary mixture of incompletely miscible components is usually used. Most frequently one of the components is water. The developer is then made up, in most cases, by shaking the water with the other (organic) component (in a separatory funnel) long enough to reach saturation of both phases and letting them separate. It is important that separation be complete, and that no emulsion of one phase remain in the other. The organic-rich phase is then used as the developer, and the water-rich phase is placed in a dish or beaker in the chamber, if it is a large one, to facilitate saturation of the air space around the paper. This procedure is an easy way of ensuring that a developer of fixed composition will be obtained every time it is made up, provided

TABLE VII-2

Filter Paper Characteristics[a]

Type[b]	No.	Texture[c]	Solvent boundary[d]	Uniformity[e]	Weight (mg. sq. cm.)	Solvent speed (min.)[f]	Ninhydrin color[g]	Resolving power[h]
S.&S. 589 blue	1	B	A	A	11	260	Blue gray	9
S.&S. 507	2	A	A	A	11	240	Blue gray	10
S.&S. 589 red	3	B	A	A	11	180	Blue gray	11
S.&S. 602 E.&D.	4	B	A	A	11	270	Purple	12
Whatman 1	5	B	A	B	11	190	Purple	12
S.&S. 602	6	B	A	A	11	280	Pink purple	13
S.&S. 576	7	A	A	A	11	280	Blue gray	14
Munktells 0	8	C	A	B	8	60	Pink rose	14
S.&S. 598 YD	9	B	A	A	13	100	Pink	15
E.&D. 7	10	B	B	C	9	120	Pink	16
Munktells IF	11	D	B	B	9	80	Pale Pink	15
E.&D. 248	12	D	C	B	11	240	Purple	13
E.&D. 613	13	D	C	A	9	180	Pink	15

[a] From Rockland, Blatt, and Dunn (785).

[b] S.&S., Schleicher and Schuell; E.&D., Eaton and Dikeman.

[c] A, smooth; B, medium rough; C, rough; D, very rough.

[d] A, even; B, uneven; C, very uneven.

[e] Based on per cent transmittances, each the average of four values determined with a photoelectric colorimeter (Lumetron 402EF) over four different areas of a trapezoidal strip of filter paper. Values found were S.&S. 598 YD 43, E.&D. 248 46, S.&S. 589 blue 47, E.&D. 613 58, E.&D. 7 60, and all others 49–54. Mean deviation from mean values, A, less than 1%; B, 1 to 2%; C, more than 2%.

[f] Time required at 26° for water-saturated phenol to ascend 120 mm. on trapezoidal filter paper strips. Each value is average of 5 to 10 closely agreeing replicate determinations.

[g] Most common color observed for chromatograms of individual amino acids stained by spraying paper strips with 0.25% ninhydrin in water-saturated butanol and heating sprayed strips for 5 min. at 80°C.

[h] Ratio of length of stained amino acid chromatogram to distance traveled by solvent boundary. Each value is average of values found for 12 different amino acids each spotted with 10^{-4} ml. of 0.03*M* solution of trapezoidal filter paper strip.

TABLE VII-3

Some Properties of Papers

Paper		Avg. thickness (mm.)	Avg. wt. dry paper/area dry paper (mg./cm.)	dh/dt for dist. water at 6 cm. (cm./min.) (machine direction)	Time for water to rise 18 cm., (min.)
W.	1	0.16	8.4	0.32	—
W.	5	0.22	9.2	0.17	158
S.S.	507	0.095	7.8	0.23	122
	598B	0.17	8.6	0.39	65
	595	0.15	5.7	0.55	49.3
	597	0.18	7.8	0.71	38.1
	598	0.36	14.3	1.27	21.4
	604	0.21	8.2	1.24	22.2
E.-D.	950	0.17	8.3	0.16	173
	952	0.19	8.5	0.19	147

[a] From Ackerman and Cassidy (6).

[b] W. = Whatman, S.S. = Schleicher and Schuell, E.-D. = Eaton-Dikeman. Thickness was measured with an Ames gage, No. 25. One-, two-, and three-sheet thicknesses were measured for each paper at four places and calculated to average single-sheet thickness. The rates of rise were determined at 25.1°C., except W. 1, which was done at 24°C., and represent the averages.

TABLE VII-4

Some Filter Papers That Have Been Suggested for Chromatographic Use

D'Arches No. 302, a French paper (529)

Eaton-Dikeman, No. 7 (0.0065 in. thick, capillary rise of water in 1 min. $^{21}/_{16}$ in.); 048 (0.007 in. thick, $^{10}/_{16}$ in. rise); 613 (0.006 in. thick, $^{14}/_{16}$ in. rise); 629 (0.012 in. thick, $^{21}/_{16}$ rise)

Japanese paper No. 2 (Toyo) (490)

Munktells Swedish Filter Paper, S-18850, double acid washed, specially developed for chromatographic use; S-18860, a similar but thicker paper (E. H. Sargent & Co.)

H. Reeve Angel Nos. 201; 204; 211 (494)

Schleicher & Schuell, Nos. 507; 589 Blue, Red, White, & Black Ribbon; 595; 598; 602 (494)

Whatman, Nos. 1 (medium rate); 3MM (thick, medium rate); 4 (fast); [illegible] (fast, acid washed, thick); 54 (a hardened version of No. 4) (27) (H. Reeve Angel)

Notes: Cellulose powders are made by Schleicher & Schuell and by Whatman. Sargent provides a trial package of 2 sheets each of 16 Whatman papers.

Schleicher & Schuell produce papers impregnated with infusorial earth (No. 287); "activated carbon" (No. 508); a black paper (No. 2490 Black); a glass-fiber paper (No. 24); and "barium-impregnated" strips (No. 85 strips).

Some of these firms are making experimental papers of various kinds of fibers. In this group can be listed E. I. du Pont de Nemours & Co., and H & V Specialties, Inc., and H. Reeve Angel. Papers made of asbestos, Dacron, glass, nylon, Orlon, quartz, and Teflon have been reported.

The addresses of the U. S. firms above can be found in the appropriate Appendix. This table is meant only as a convenience for the reader. Nothing that is said implies any recommendation of these products. It must be cautioned that there are over 75 grades of filter papers available, and possibly there are other manufacturers in this country (United States) not known to the author, and other papers that are available

that the equilibration is always done at the same temperature, or that the composition of the resulting mixture is not very temperature-sensitive (191). When, however, the composition of the saturated developer phase is sensitive to the temperature at which it is made up, it is best not to use a saturated solution (137,246).

For example, when water-saturated phenol is used as developer, if the temperature were to fall in the chamber (as might happen at night), the phases might separate, and the chromatogram be ruined by waterlogging. For this reason, and also for ensuring reproducible R_F values, Draper and Pollard (246) recommended 25% water in phenol, for amino acid separations, and Bull and co-workers (137) recommended 20%. These solutions, which are undersaturated at room temperature give more reliable results in the absence of temperature control. On the other hand, water becomes more soluble in *lutidine* (689) and *collidine* at lowered temperature, so waterlogging is not so likely here. However, lowered temperature, understandably, because of this raises the R_F of amino acids in collidine (581).

Ternary and quaternary developers would be expected to be more sensitive to temperature change than binary ones. Bate-Smith and Westall (43) found that the system 1-butanol: acetic: water is very sensitive to temperature change. The developer is made by mixing the liquids in the ratio 4:1:5 by volume, and letting stand for 3 days at constant temperature. The organic layer is used. These workers recommend that the temperature be controlled to ± 0.5° C. They also state that the developer is sensitive to concentration change through differential evaporation and hence can be used satisfactorily only in a tight system. Similar difficulty may be encountered with systems using such volatile components as ether or petroleum ether. Running these chromatograms in a cold-room is advisable. It is sometimes recommended that the mixtures be allowed to stand for a period of time before being used: for example, for 1 hr. (502) or 3 days (43).

With multicomponent developers, it is good practice to check the composition of different batches and of the same batch from time to time. This may conveniently be done in some empirical way, such as by cooling or warming the mixture and reading the temperature when cloudiness just appears. This is an exceedingly sensitive test of composition. Another approach is to add a known excess of water to a measured amount of the mixture, or a known quantity of salt, or salt solution, in a graduate, and measure the volume of organic phase that is released.

Developers containing several components may show frontal analysis phenomena (see below), even though the paper has been thoroughly conditioned in the vapor of the developer. (An observation interesting in this

connection is that cotton equilibrated with liquid water took up twice as much water as it did from water vapor (525). This implies multilayer formation on the fibers, and has implications for the problem of conditioning paper. For example (among a wealth of examples) Lederer (530) observed, in a study of the chromatography of the noble metals, that a developer of butanol saturated with *N* hydrochloric acid, rising up Whatman No. 1 paper, produced two fronts. The upper front was of a dehydrated butanol. Several centimeters below this there was a darker, aqueous front. This latter was used for calculating R_F values; hence if a zone ran in the dehydrated butanol region, its R_F value would be greater than 1 by this convention.

Bate-Smith and Westall (43) made a similar observation with a developer separated from a butanol:2*N* HCl (1:1 by volume) mixture. The lower, hydrochloric acid front showed a position between 0.8 and 0.9 in terms of R_F. Linstead and co-workers (143) and others have noted more than one front on the paper. For example, a pH gradient has been observed in a phthalate buffered strip (from pH about 4.8 at the top to pH about 3.8 at the bottom, in downward development with isopropyl ether–isopropyl alcohol developer). The influences of these gradients upon R_F values will be discussed below.

Some developers are made to contain quantities of acid, or base, for the purpose of swamping the ionization of some substance that is to be separated. This makes it possible to drive the substance out of an aqueous into an organic phase. For example, it is common practice to add formic acid to developers used to separate dicarboxylic and polycarboxylic acids. While formic acid is not so strong (highly ionized) as some dicarboxylic acids (for example, the first ionization of oxalic, malonic, maleic), nevertheless it has a sufficient suppressing effect (Chapter XIV) and, moreover, can later be removed from the paper by steaming.

Developers are sometimes designed to exert other chemical effects. For example, in the separation and estimation of vat dyes and related substances in the leuco (reduced soluble) form, reducing developers are necessary. Midgelow (634) used Cellosolve-hydrogen sulfite solution containing sodium hydroxide; Rao and co-workers (758) and Klingsberg (481) used a solution consisting of aqueous sodium hydrosulfite in tetraethylene pentamine. This was said (481) to be superior to the caustic alkali solution. It consists of 10 g. sodium hydrosulfite in 100 ml. of aqueous amine. This solution is prepared as needed from the solid hydrosulfite and a stock solution consisting of 100 ml. amine in 1 l. of water.

Borate has been used in a developer to retard the movement of *cis*-diol sugar ester zones (188) and the same principle has been used to immobilize ribosides and to separate them from related and unrelated

contaminants (789). This kind of use of a developer can be broadly generalized, as shown below.

When another developer is to be used on the same chromatogram it is almost always necessary to remove all traces of the first. For example, if the first is phenol, and is to be followed by collidine, then at the end of the phenol development the paper is dried until it no longer smells of phenol—some workers flood it with ether to remove last traces of phenol (if the substances being separated are not soluble in ether)—and the part of the paper that dipped into the developer is cut off, as well as the other edge of the sheet that contained the front. The front is usually cut off because impurities washed out of the paper collect there (sometimes as brown streaks, or "fingers"). Only then is the sheet turned through 90° and developed with the second developer.

A variety of different ways of handling repeated development have been used. The same developer may be used repeatedly, "multiple development," the chromatogram being dried in between runs (448). This tends to crowd the faster zones into the high R_F region, but is useful with slow-running substances. With two different developers run in the same direction, as with two-dimensional chromatography, the first must be removed. For example, Pollard and co-workers (740) found a "one-way two-solvent" method useful in some inorganic separations. They first chromatographed with a butanolic developer, then washed the paper with chloroform and dried it, then used a dioxane developer in the same direction. The two different developers might contain different complexing agents. They also reported a "two-way three-solvent" separation, using a collidine developer as the first, followed, after drying, by a butanol developer run at a right angle to the direction of the collidine run, and after washing and drying a dioxane developer run in the same direction as the butanol one.

The discussion of developers has dealt largely with conventional paper chromatography. It can, *mutatis mutandis*, be extended to the reversed phase type and to column partition (Chapter VI).

VI. ZONES OR SPOTS. BANDS

In general, in a good chromatogram, the zone of a separated substance should be regular in shape, with a well-defined boundary, and well separated from the zones of other substances. This means that the zones of the different substances should move compactly, and at quite different velocities. The factors that influence these behaviors are discussed in a special section, below. Here we treat chiefly of the properties of the zones as they are observed before and after chromatography. We will discuss strips and sheets first, leaving any special features of the radial method until the end of this part.

The initial mixed zone may be applied to the paper either as a circular spot, or as a long streak, or band, extending usually from edge to edge of the paper. Rarely are other shapes used. The zone is usually applied to the dry paper before it is placed in the chamber. Two requirements need to be met. The zone should be compact, and for reproducible R_F values the zone should always be placed at the same distance from the surface of the developer (191).

The zone can best be applied with a micropipette or microburette, the tip of which has been wiped after it has been filled. Ulteé has described a method for limiting the size of the zone. A ring of a mixture of 52.5% hexachloroethane and 47.5% naphthalene is deposited in the paper from the end of a fire-polished glass tube of appropriate size. The sample is deposited inside this ring, and after it has dried the hydrocarbon mixture is removed by sublimation at 50°C. (951).

The size of the zone may be limited by adding the dissolved substance in small repeated portions of solution to the paper, using a current of dry air or the heat of a lamp to hasten drying. In this way quite a large amount of substance can be placed on a small area of paper.

Other methods, applicable to concentrating a solution into a small spot or to transferring a zone from one chromatogram to a position on another, have been suggested by numerous investigators, among them Consden, Gordon, and Martin (192), Dent (225), Moore and Boylen (647), and Gregory (349). Among the methods used is: to concentrate the (aqueous) solution of substance, resting as a drop on a nonwetted surface (a sheet of polyethylene or Teflon) by gently blowing air over it until it is decreased in size to the proper degree, then transferring it by touching the paper to it (192). Another procedure is to transfer the substance by upward or downward elution, or by capillary flow from a small container, through the tip of a pointed piece of paper, as shown in Fig. VII-13 (349,647).

The spot should be placed on the paper at a location marked lightly with a lead pencil. This marked point serves after the zone has moved away as a reference point for measuring R_F values.

Actually the point of application should not be too far from the surface of the developer—about 1 to 1.5 cm. is usual. It must be a little distance away from the developer because the first rush of developer into the paper is quite turbulent, and may widen the zone out at right angles to the direction of development if it reaches it (495). The most important requirement about the location of the initial zone is that for reproducible and comparable R_F values *it should always be at the same distance from the surface of the developer.* This was recognized in their first publication by Consden, Gordon, and Martin (191). Kowkabany and Cassidy (495)

showed that the R_F's of some amino acids are essentially linearly related to the distance of the initial spot from the surface of a mixed developer (Fig. VII-14). The reason appears to be that not only is there a mass gradient along the developed paper to the front, but perhaps more importantly there is a concentration gradient (191) produced by the sorptive abstraction of water by the paper from the developer which therefore becomes more lipophilic (less polar) towards the front. There is, in other words, frontal analysis of the developer. The further the initial zone from the surface of the developer, the less polar the liquid that first reaches it (because of this frontal analysis), and the less like the developer in the trough.

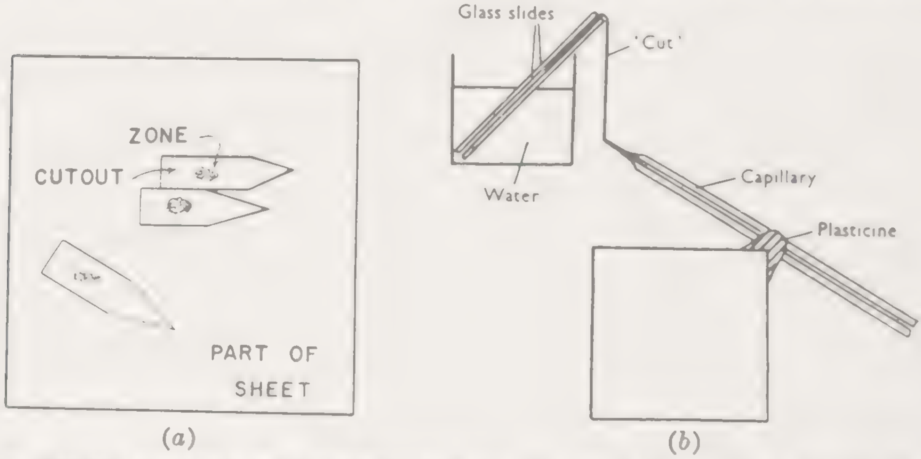


Fig. VII-13. Methods for transferring spots, or zones, in paper chromatography. (a) A tapered piece of paper containing the spot is cut out (349). (b) This is connected with a source of eluent which washes the spot countercurrently by capillary flow of the eluent. The effluent from the tip of the paper is either collected in a capillary tube (b) (192) or put on a piece of paper (647) often with simultaneous drying for further chromatography. The liquid may be expelled from the capillary onto a piece of polyethylene or Teflon (which it does not wet if it is aqueous) and subjected to concentration, or other manipulations, or it may be reacted within the capillary (192).

More polar components are therefore not moved as readily by this depleted front, and they begin to pick up speed only as the front moves on and the concentration of the developer reaches a steady state identical with the bulk concentration of the developer. Thus, with amino acids and normal chromatography in general, the further the initial spot from the surface of the developer, the lower the R_F value. Moreover, the R_F changes with time at first, being lower at an early stage of development than later. So it turns out that up to a point the R_F value increases with the distance moved by the front. This effect is more noticeable when the paper has not been conditioned; however, it does not seem to be entirely removed even on long conditioning (495).

Although the initial zone should be made as compact and well defined as possible, the paper should not be overloaded. *The most common mistake among beginners is to overload the paper with mixture.* It is better to use a larger zone (and wider paper strip, or fewer zones) than to lay down a small overloaded zone.

By overloading is meant that there is more solute than can be carried by the mobile phase that will cover the area of the zone. Overloading

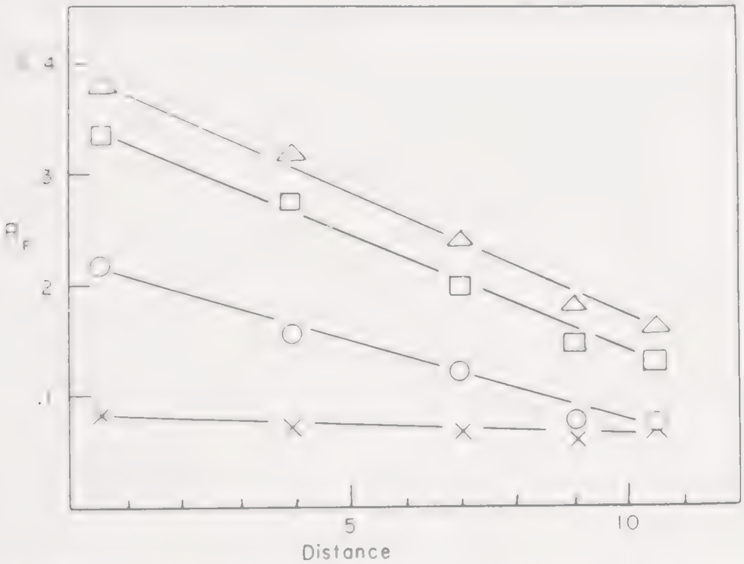


Fig. VII-14. Change in R_F value with distance of initial spot of amino acid from surface of developer, and with mole fraction of collidine in aqueous developer. R_F values on the ordinate are plotted against distance in centimeters that an initial spot of valine was placed above the surface of collidine–water developer. Each curve represents a different mole fraction of collidine: Δ, 0.19; □, 0.21; ○, 0.37; ×, 0.52 (mol fraction calculated on the basis $C_8H_{11}N$ for a collidine fraction boiling between 46° and 71°C. at 19 mm.). (From Kowkabany and Cassidy (195).)

causes streaking in the direction of flow of the developer, and some lateral widening of the zone. The latter is caused by accelerated diffusion from the region of high concentration, and is a manifestation of a process that goes on also in the direction of flow. The streaking may have several contributing causes. At first the flow of developer is quite rapid, later slowing down considerably in upward development. This will not be conducive to the mobile phase carrying a maximum (equilibrium) amount of solute. Secondly, the frontal analysis may be marked enough in the leading region of the developer to markedly decrease the solvent power of the liquid for the solutes (in the case of amino acids and a water-collidine developer). Both of these factors would operate, if there were

an excessive accumulation of solutes in the initial zone. So the material of the zone would be moved by a gradual leaching process, which seems to be a cause of tailing. An oversupply of solutes in the initial zone would cause tailing in the absence of the above factors. Other possible causes of tailing are discussed below.

Tailing *due to the above causes* can be avoided by using a slower paper, or a thicker paper, or a lower concentration in the initial zone, i.e., a larger zone for the same amount of material, or a smaller zone with less material. An ordinary paper chromatogram may handle, roughly speaking, up to 50 μg. of a component in a zone, but usually (if the detection methods are good enough) it is better to use 1 to 5 μg.

Sometimes the material that is to go into a mixed zone can most conveniently be formed by a reaction in situ on the chromatographic paper. For example, Williams and Bevenue (999) were able to verify raffinose and sucrose (10 to 50 μg. of each) by spotting 1 μl. of sugar solution on paper, superimposing 5 μl. of invertase, and letting stand 5 min. The hydrolysis was then complete, and on developing the zone with butanol: ethanol:water (10:1:2), the resulting melibiose and levulose from the raffinose and dextrose and levulose from the sucrose, moved to the same extent as pure sugars run in parallel as controls.

Another example is given by Kream and Chargaff (497). When they had available only small quantities of substrates or enzymes which they wished to react together so that the products of the reaction might be analyzed and identified chromatographically, they reacted the two directly on the paper. The substrate was spotted and then rapidly overlaid with enzyme. The paper was incubated in a moist atmosphere for the necessary length of time, the spots being in some cases remoistened from time to time. After incubation the paper was heated in an oven at 90° to 100°C. for 15 min. to stop the action of the enzyme and then chromatographed in the usual way.

VII. CONDITIONING

After the initial mixed zone has been applied to the paper it is placed in the chamber in the presence of developer and/or the other phase (the more polar phase in conventional types of chromatography; the less polar phase is reversed phase chromatography). Development is not begun for a period of time (the end of the paper is not dipped into the developer). During this period the paper sorbs the components of the vapor phase of the cabinet, and so becomes conditioned (6), or accommodated (831), or "equilibrated." Long conditioning may not be necessary with small strips in test tubes, except for precise work, but it is advisable in all work as a matter of principle.

Conditioning has the following effects. When the paper is placed in the vapor of a liquid such as water, it takes it up rapidly in the first 30 min., then more slowly, but even after many days it may still be taking on water (668). The amount eventually taken up from saturated water vapor may be 20% by weight (816). When strips of Whatman No. 1 paper (oven-dried at 80° to 85°C., overnight) were hung in an atmosphere over collidine–water, at 23.0° to 23.8°C., the strips gained weight relatively rapidly for 8 to 12 hr., then more slowly. After 48 hr. they had gained about 17%, based on the initial weight of the dry paper, and were apparently still slowly gaining. When strips of the paper were immersed in the liquid developer, the changes in refractive index indicated preferential adsorption of water, particularly in liquids where the water/collidine ratio was higher (495).

These data and others indicate that the paper preferentially takes up the more polar substance present during conditioning. This should have the effect of lessening the frontal analysis that would occur if the same developer were run on unconditioned paper. It is found, indeed, that with prolonged conditioning the effect on R_F of moving the initial zone further from the surface of the developer (see above) is lessened. Holding the paper momentarily in a jet of steam so that it becomes moist but not wet has the same effect on this phenomenon as long conditioning with an aqueous developer does.

Shute (831) has analyzed the process of conditioning, or accommodation and its effects, essentially, as follows. His work was done partly with single, and partly with binary, development. If paper is not conditioned, then since the process of conditioning that involves imbibing of the cellulose is relatively slow, the ratio of V_S/V_M will not immediately have the value it would on conditioning. Here V_S and V_M are the *volume* phases of the stationary and mobile phases, respectively, and are related to the areas A_S and A_M. Therefore R_F will be affected. He had shown previously that when alkaloids are chromatographed from aqueous ammonia the R_F decreases on conditioning the paper. Thus atropine, developed on Whatman No. 1 paper with 5% ammonia, showed an R_F value of 0.90 when the paper had been placed into the saturated vapor and developed immediately. After 95 hr. of conditioning the R_F found was 0.56. Again, glucose, developed with water, showed an R_F of 0.90 without conditioning, and 0.66 after 40 hr. conditioning. When antipyrine and pyramidon were chromatographed with butanol saturated with water R_F values of 0.82 and 0.87, respectively, were found without conditioning, and 0.74 and 0.76 after 80 hr. conditioning.

He points out that the decrease in R_F can be derived by considering the change in volume of the stationary phase (it would be expected to increase

relative to the mobile phase) on conditioning, assuming α to be constant

$$dR_F = [-\alpha/(1 + \alpha V_S/V_M)^2]dV_S/V_M$$

Now $\alpha/(1 + \alpha V_S/V_M)^2$ has a maximum for the value $V_S/V_M = 1/\alpha$ which corresponds to $R_F = 0.50$. Then, the decrease in R_F laid to a given increase in V_S/V_M will be greatest when the R_F is 0.50. He concludes, also, that conditioning that involves imbibition will improve separations of substances with $R_F > 0.50$. This was observed by Schute in several cases, and he refers also to improved separations obtained by Wieland and Feld (994) upon conditioning the papers. This subject is further discussed below.

VIII. DEVELOPMENT

When the paper has been conditioned for the appropriate period, it is dipped into the developer or, in downward development, the developer is run into the trough. After the development has proceeded long enough, which may be until the developer has nearly reached the extreme edge of the paper, or in other cases, has progressed a certain distance previously known to be sufficient, or in still other instances, after the developer has run off the sheet for a sufficient period of time, the paper is withdrawn from the chamber and dried in a ventilated oven or in a hood with good draft. It may be run again with the same developer (448) or subjected to the various techniques described above.

Attention should be paid by the chromatographer to the fact that many of the developers used in chromatography are toxic. The Merck Index of Chemicals and Drugs, 6th edition (1952), gives information on toxicities. For example, Mr. Joseph Shapiro has called my attention to ethylene chlorohydrin which may be used in a developer for phospholipids and peptides. According to the Merck Index, inhalation of the vapor has been fatal; poisoning occurs from inhalation of 18 p.p.m.; and the maximum allowable concentration probably is below 1 p.p.m. The volatile chlorinated solvents are in general toxic, and should be treated as dangerous chemicals. The maximum allowable concentration of chloroform for a prolonged period is said to be 100 p.p.m. Phenol, collidine, lutidine, butanols, and the amyl alcohols are to be treated as toxic substances. It is recommended that the chromatographer acquaint himself with the physiological properties of his developers.

IX. REVELATION OF ZONES

Paper chromatography is used so commonly with colorless substances that a large literature has grown up on the devices that may be used to

reveal the zones after development. These are discussed for all kinds of chromatography in Chapter XI. Whatever device is used to reveal the zones, it is common to characterize them by their R_F values.

X. DETERMINATION OF THE R_F VALUE

The R_F value has already been described in Chapters II and IV. In their first detailed paper Consden, Gordon, and Martin (191) pointed out that best results were obtained (elimination of variability caused by the paper) by measuring the distance moved by the zone from the point of application to the center of density of the zone. They pointed out also that there was some variability among R_F values, even among duplicate runs made simultaneously in the same chamber. This situation has been improved somewhat, as it has been learned what factors may cause variability and how to control them. Thus in their quantitative work Bate-Smith and Westall (43) could recommend discarding all papers in which control substances yielded R_F values that varied more than ± 0.02 from the standard value for the control.

Such precision is not usually demanded in ordinary work, *though it is highly desirable*. An R_F value, in which the substance being examined is run along with one or more known reference compounds, and the relative position is judged, is a more reliable criterion of the substance than an R_F value determined uniquely from a table Relative R_F values, that can be deduced from tabulated results, are more valuable, therefore, than the individual values themselves. For this reason the *pattern* of a chromatogram is important for characterizing the mixture that has been separated.

Because of the reliability of relative R_F values, several have been defined, two of which are

$$R_G = \frac{\text{distance traveled by the substance (usually a methylated sugar)}}{\text{distance traveled by tetramethyl D-glucose}}$$

These distances are measured from the starting line to the centers of the zones (409).

$$R_T = \frac{\text{movement of steroid}}{\text{movement of standard steroid}} \times \text{(rate of movement of standard) (810)}$$

The use of relative R_F values has been extended by Partridge and Westall to help correct R_F values determined at other temperatures to a standard temperature. The R_F value, determined at a temperature $t°$, is multiplied by a factor a, previously determined for various values of t.

$$a = \frac{R_F \text{ value of glucose at } 20°}{R_F \text{ value of glucose at } t°}$$

According to these authors when R_F values determined (with a given batch of paper) at different temperatures are corrected to the standard 20°C. greater constancy is observed between the values for any given sugar. This should aid identification (710).

Various other measurements related to R values have been defined. For example, Evans and co-workers (275) find an R_F' value useful. This is defined:

$$R_F' = \frac{\text{distance from origin to leading edge of spot}}{\text{distance from origin to front of developer}}$$

R_P, a measure of "resolving power," is defined by Rockland, Blatt, and Dunn as (785)

$$R_P = \frac{\text{length of zone of amino acid}}{\text{distance traveled by solvent boundary}}$$

The R_M value will be discussed in Chapter XIII.

When the solvent front runs beyond the paper, it becomes necessary to use relative values. In such a case a standard of known R_F value may be run. Then, from the distance it has moved one may calculate where the front would be if the paper had been long enough, and thus the R_F's of the other substances can be calculated. Or, R_F of "unknown" = (R_F of reference substance × distance moved by "unknown") ÷ distance moved by zone of reference substance. The need for such a relative value in the long runs required to separate certain steroids led Savard to define R_T (above) (810).

The R_F value is not usually considered sufficient to identify a substance in a zone. If it is at all possible three other kinds of evidence are sought. (See the discussion of purity in Chapter VIII, Section X). An authentic sample of the known substance is run on the same paper alongside the other that is suspected to be identical with it, and the two zones must show the same R_F value; the authentic known is added to the mixture containing the suspected material and must not be separated from it. The zone of substance should show its original R_F, and only be larger in area or deeper in intensity of color. An extension of this latter test, used with radioactive zones, is to prove that the zone of radioactivity in a known sample exactly coincides with the color zone of the substance suspected to be identical with it. The third bit of evidence requires running these tests in a different solvent system, preferably differing quite a good deal in polarity (though this may in practice be difficult to achieve), but at least, a different solvent system. The first two tests should then again be met, whereupon it is very likely that the known and the other substance are identical.

What has been said so far about zones largely applies also to radial chromatography. There are some differences, of course, between radial and longitudinal development. In the radial method the component of a zone is continually spread along over a larger circumference as the zone moves out toward the periphery of a disk. Wood and Strain (1022) indicate that under certain conditions the trailing (inner) edge of the zone may move faster than the leading edge, in which case the zone would become narrower as it moves outward, the concentration of solute in solution in the zone tending to remain constant. Also, the mass gradient of developer in the paper is quite marked, according to these authors.

The R_F in radial chromatography is determined as the ratio of the radial distances from the center of the initial zone to the center, or place of greatest density of the developed zone, and to the front of the developer. Saifer and Oreskes (800) point out that because many measurements can be made of the R_F of a single zone—along different radii—it is possible to achieve a high accuracy. On the basis of six measurements, the mean could be determined with a standard deviation of ± 0.02 R_F unit—a requirement also made upon quantitative work by Bate-Smith and Westall (43). The different measurements take account of the differences in liquid flow due to fiber orientation in paper, and so lead to high reproducibility of R_F value between different runs, according to these authors.

XI. RECOVERY OF SEPARATED SUBSTANCES

In some analyses, as of peptides, it is necessary to remove a zone from a paper chromatogram. Consden and co-workers (192) showed how this might conveniently be done by cutting out the zone in a slip of the paper, making a point at one side, and washing off the zone with some eluting agent, countercurrently (see Fig. VII-13). Dent's device (225) can be used conveniently. Countercurrent elution is always preferable to batchwise elution in a beaker or test tube.

XII. PRESERVATION OF DATA

When it is desired to keep a record of the pattern of the chromatogram the circumferences and centers of density of the zones should be marked in pencil. Many of the colors produced by agents used to reveal spots are not stable, or the reagents eventually discolor or actually decompose the paper. Ninhydrin colors tend to fade, but can be stabilized by the method of Kawerau and Wieland (463). The papers, having been treated with ninhydrin and heated to bring out the colors, are sprayed with 1% copper nitrate in ethyl alcohol. The spots appear salmon-pink on a pale bluish

green background. Levy and Chung (554) find that this treatment prevents fading, and by improving contrast, allows better photographs, or contact prints to be made of the papers. They can also be more freely handled without soiling. The chromatogram may then be sprayed with a lacquer.

Many chromatographers preserve their results by photographing the chromatograms or by making direct contact prints on a paper like Kodagraph Standard contact paper (Developer D-72) (554), or by using a reflex printer such as the Contoura. It may be noted that some reagents cause chemical development of a film or paper on contact.

As in the previous sections the special features of reversed phase chromatography can be derived from what has been said about the ordinary methods.

XIII. METHODS

The goal of chromatography is to accomplish a separation of the mixture in hand. In order to attain this goal in paper chromatography at least five ideal conditions should be approached.

1. The zones of the components of the mixture must move at different and reproducible velocities in a straight line in the direction of motion of the developer. Reproducibility rarely implies absolute reproducibility, for this is at present difficult to ensure because of uncontrollable and some probably unrecognized variables in the system. Relative reproducibility, meaning that the ratios of R_F values of the components chromatographed are reproducible, is easier to ensure. A given sheet of paper which has passed visual inspection for gross faults is more likely to behave in a homogeneous manner over its surface than are two different sheets to behave identically, and in a single run the variables other than paper—the temperature, developer concentration, presence of contaminants—are likely to have the same effects on all parts of the paper if ordinary precautions are taken. The value of relative R_F's was discussed above. The requirements for running in a straight line at a reproducible velocity are related to the calculation of R_F. Swerving makes determination of R_F difficult, and may distort the shape of the zone; also a swerved zone may interfere with a neighboring zone.

2. The zones must retain regular, compact form, preferably circular, and if elliptical the ratio of the axes should not be far from 1, except that the farther apart the zones in the developed chromatogram the greater the elongation that may be tolerable if at the same time the boundaries remain reasonably sharp and the contours reasonably regular. With irregular or overelongated zones it may be difficult to determine the point of maximum density to use in measuring R_F value.

3. *It must be possible to find the zone in some way* (Chapter XII).

4. *It must usually be possible to detect the front of the developer in some way*, unless, as described above, a reference substance is used and relative R_F is determined.

5. *The developer must flow along the paper at a reasonable velocity.* By reasonable is meant a velocity slow enough that the components of the zones can distribute between the stationary and mobile phase to a nearly maximum efficiency, but not so slow that diffusion effects become pronounced. For small strips, *ca.* 13 cm. long, as in the Rockland and Dunn method (787), a running time of 20 to 180 min. is reasonable; for long strips, in which the solvent front travels 40 cm. or so, "overnight" is a reasonable time. (If there is no other way of solving the problem, "several days" may be reasonable.)

1. Examples of Paper Chromatograms

The following methods are described to show how typical paper chromatograms may be run. They illustrate a simple, rapid qualitative method that is especially useful for screening developers; a method using a strip, with very long development; a two-dimensional method; radial methods; a frontal analysis; and a gradient analysis.

A. ROCKLAND AND DUNN METHOD (785,787)

Paper strips are prepared by cutting from a sheet, or roll, in the machine direction, pieces 13 cm. long and 1.8 cm. wide at one end, 1.0 cm. wide at the other. These are designed to fit standard 6-in. test tubes in such a way that when the bottom of the strip rests in the bottom of the test tube the upper edge rubs the walls enough to keep the paper upright. (See Fig. VII-1.) It is convenient to make a metal or cardboard model of a strip and use it to sort out a batch of test tubes of the same size. This template may be used also as a pattern to cut the papers. Better, a pattern composed of ruled lines may be drawn up, mimeographed, and used as a master for cutting batches of strips at once. The dimensions given above may be modified to fit larger or smaller test tubes, but whatever size is used the strips should be cut cleanly; otherwise, developer tends to creep unevenly and more rapidly up rough or frayed edges than up the main body of the paper. Mackay (592) cuts strips of the right length from a roll of filter paper and tapers the last few centimeters (Fig. VII-1). It appears that once the first rapid rise of liquid has occurred, there is little further danger of capillary rise of excess liquid along the paper where it touches the wall of the tube, and these strips function perfectly well.

With an ultramicroburette, or other suitable device, the mixture is

applied at the center of the paper on a spot marked lightly in pencil 0.8 cm. from the narrower end. A volume of 0.15 μl. is about right. This dries in about 1 min., and a number of aliquots, as when a mixture is being produced, or when the amount of material is being built up, may be successively placed on the same spot. A current of warm air directed upon the spot is advantageous.

The test tube to be used stands vertically in a rack and contains developer to a depth of 3 to 4 mm. It should be tightly stoppered, preferably with a glass stopper, though quite satisfactorily with a softened cork or foil-wrapped cork. It should be shielded from drafts, steam pipes, light bulbs, or other sources of temperature gradients.

The paper is inserted in the tube and may advantageously be allowed to remain suspended above the developer for a while—$^1/_2$ to 1 hr.—for purposes of conditioning, though in many cases it is immediately pushed gently down into the developer so that the narrower end is immersed to a depth of 2 mm.

After the developer has risen the desired distance up the strip, the paper is removed from the tube, the position of the front is marked either in pencil, or by snipping the paper at the edges with a scissors, and it is hung in the hood to dry. A satisfactory distance of rise is 10 to 12 cm., that is, 9.4 to 11.4 cm. beyond the initial spot. The air-dried strip may then be further dried in an oven or sprayed with the revealing reagent directly.

Time required for an experiment varies greatly with the liquid. Thus, for example, to rise 10 cm. above the initial spot (10.6 cm. above the surface of the developer), the following liquids required approximately the times given (in minutes): methylethyl ketone, 12 to 14; diethyl ketone, 14 to 15; toluene, 15 to 17; dry benzene, 17 to 18; octane, 17.5 to 18.5; chloroform, 19 to 21; *sym*-collidine, 24 to 25.5; water, 25 to 30; carbon tetrachloride, 30 to 35; absolute alcohol, 42 to 46. To rise 7 cm. from the initial line, *n*-butyl alcohol required 42.5 to 50 min; sec. butyl, 48 to 57 min.; *iso*butyl 55.5 to 65.5; and tertiary butyl, 85 to 105 min. The rate seems to depend most importantly on the viscosity of the liqiud (6).

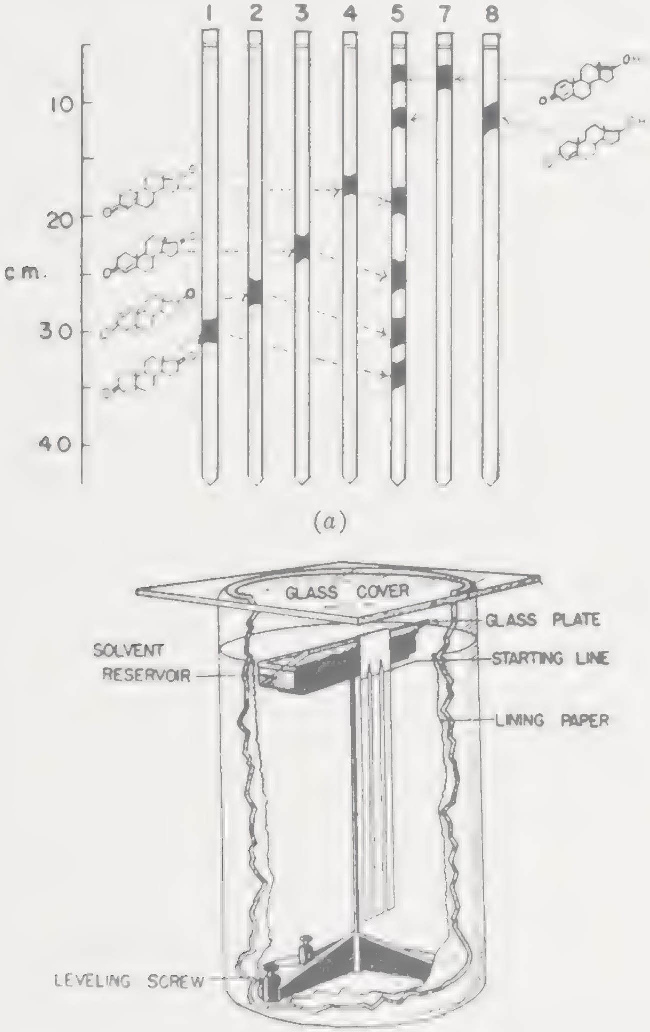
Rockland and co-workers have described a number of mechanical devices for facilitating this method (785).

B. STRIP METHOD (SAVARD)

An example is taken from Burton, Zaffaroni, and Keutmann (147), as modified by Savard (811), and used with steroids by these authors.

Strips of filter paper (Whatman No. 1 is used) are extracted in a Soxhlet

apparatus with alcohol, and benzene, and dried. They are then cut into separated sections 1 × 45 cm. long, attached to each other through an upper strip as shown in Fig. VII-15*b*. The strips are then impregnated with stationary phase (for example, propylene glycol) by dipping them into fresh 50% solution of the glycol in methanol and blotting out the excess liquid. The amount of stationary phase left in the paper affects the mobilities of the steroids (but not the R_T's, which are relative values),



(*a*)

(*b*)

Fig. VII-15. (*a*) Chromatographic separation of derivatives of androstanedione and testosterone differing in the presence and location of a double bond. The solvent system is ligroin-propylene glycol. Fifty micrograms of steroid was used and the zones were visualized by Zimmermann reagent, or dinitrophenylhydrazine, or both. (Redrawn from Savard (811).) (*b*) Method of supporting the strips, which are fastened together at the upper ends. From Burton, Zaffaroni, and Keutmann (147).

so the paper is kept between blotters with the starting lines and upper end projecting while the mixture is applied. The steroidal substances or mixtures in benzene, or preferably in methanol, are applied along the starting lines, from edge to edge of the strips, in small aliquots. The methanol solvent is preferred because it tends to sweep back the glycol from the line, the back pressure of glycol tending to reduce the width of the initial zone. From 10 to 100 μg. of sterol might be applied to a 1 cm. strip, and even up to 0.5 to 1.5 mg. of mixtures. Using strips up to 16 cm. wide as much as 30 to 40 mg. could be accommodated. The limit seemed to be that which would yield a final concentration, after separation, of 1 mg./sq. cm. for a single component.

The upper joined part of the paper is sandwiched between two sheets of glass which rest in the trough of a chromatogram chamber (much as in Fig. VII-13*b*). The chamber is previously lined with paper wetted with the mobile phase and also dipping into it. Every effort is made to ensure vapor saturation. The starting line protrudes 1 to 1.5 cm. beyond the edge of the glass plate sandwich. The mobile phase of ligroin (materials in the range of 65° up to 115°C. can be used) which has been saturated with stationary phase (propylene glycol) is then added to the trough. It flows up the paper between the glass plates and on to develop the chromatogram.

After development, which may require that the strips be run for several days, with the developer dripping from the lower pointed end, the strips are removed from the chamber and dried in air (6 to 8 hr.). For preliminary examination a thin strip, about 3 mm. wide, can be cut from the edge of a strip and treated with color reagent, etc. This allows the location of zones which, when the test strip is matched to the original strip, permits cutting out the zone of original, unreacted steroid.

Because the strips have been impregnated beforehand, it is not necessary to condition them in the chamber. Because the stationary phase is practically nonvolatile, and only very slightly soluble in the ligroin, the mobile mixture cannot be relied upon (as it often is) to supply stationary phase. For the reason of lack of volatility, conditioning would be impractical. Yet the chamber must be lined and wetted with mobile phase to prevent it from evaporating from the paper. With this system, Zaffaroni and co-workers (147) use starch paste to seal the chamber, instead of stopcock or other grease.

C. TWO-DIMENSIONAL METHOD

An example of this method is taken from Block (83). On a sheet of filter paper, 18.5 × 21 in., a pencil line is drawn 1 in. from one of the

shorter edges and parallel with it. A cross mark is made 1 in. from one end of this line. It is at this point that the mixture is applied, in one or several small aliquots. For best results, Block recommends not applying more than 0.005 ml. (5 μl.) of solution at one time, and reports that up to 0.060 ml. of liquid may be applied in total without increasing the area of the mixed zone at the point of application.

The chamber is made from a rectangular glass aquarium 52 cm. long × 32 high × 26 wide, which is coated at the bottom and up a little way on the sides with paraffin (see Fig. VII-7). Three glass rods the length of the chamber are sealed to the sides with paraffin: two of them 5 cm. from the sides and the third halfway between the two and all 2 to 3 cm. down from the top of the chamber. The chamber is covered with a glass plate, weighted down. Troughs are placed on the bottom of the chamber parallel to the glass rods, and developer, 20 to 50 ml., is run into them.

The papers are draped over the glass rods, so that the edge of shorter length hangs in the developer and the spot of mixture lies just above it. The paper hangs freely without touching itself. The developer moves up the strip, over the rod, and down the other side. After it has progressed far enough, the paper is removed from the chamber, the solvent front is marked, and the paper is dried in a current of warm air (30°C.) for 6 hr. When the developer is aqueous phenol, Whatman No. 4 paper requires about 36 hr., and Schleicher and Schnell No. 598 about 24 hr. Block, in agreement with Dent, recommends running the acidic developer before the basic in two-dimensional work.

After the developer has been removed the first part of the paper that dipped in developer is cut off along the line that passes through the point of application, and the excess paper a few centimeters beyond the solvent front is trimmed off. The sheet is now turned through 90° and hung in another chamber in the same way. Now the row of zones which were produced by the first developer lies just above the surface of the second developer in the trough of the second chamber, and development takes place in a direction at right angles to the first. After development (20 to 24 hr. with lutidine) the paper is removed from the chamber, dried (about 30 min.), and treated with the color reagent.

Much smaller sheets than these can be used, but then the initial spot must be correspondingly smaller. Good two-dimensional chromatograms can be made on sheets 6 in. square, but the initial spot must be only 2 to 4 mm. in diameter.

Sheets can be used for one-dimensional runs. Again the sheet is ruled 2.5 cm. from one edge and the mixtures, reference substances, etc., to be examined are spotted beginning about 2.5 cm. from the edge, every 2 to 3 cm. along the line. In this way as many as 22 spots may be applied, with unknowns alternating with known reference substances.

With the use of deeper chambers the sheets may be hung their entire length, with two sheets to a trough, one on each side. The larger the chamber, the more attention must be paid to conditioning.

Instead of hanging the extended sheet and using downward development (or hanging it in a trough and using upward development) the paper sheet can be formed into a cylinder and stood in a layer of developer in the bottom of the chamber (421,1001). (See Figs. VII-3 and VII-7.)

A method which gives the chromatographer a great deal of control over his method, particularly through a control of the velocity of flow of the developer, has been devised by Irreverre and Martin (433). A cylinder of paper is prepared by punching holes along two opposite edges, and lacing the edges together by superimposing them and running a strip of the same paper through the holes. The punching should be done carefully so the cylinder is straight. Thus there are no metal staples present. This is stood in a glass cylinder around a stand made of two funnels, as shown in Fig. VII-10. A ring of rubber tubing or a rubber stopper can replace the Teflon ring.

The developer is fed to the cylindrical sheet through two superimposed disks of paper, through the center of which a piece of knitting cotton, knotted on the end, has been threaded. The papers rest on the top of the paper cylinder, and the wick dips into a dish of developer. By using more strands of cotton, liquid can be fed faster to the cylinder of paper. By using exactly a known amount of developer the chromatogram can be stopped at any predetermined time. The shorter the wick above the developer, the faster the flow, and also the larger the number of wicks (threaded equidistantly around the center of the paper disks), the faster the flow. The cotton used for wicks should be washed by downward development before use.

Using this apparatus the authors were able to vary the rate of development (40 cm. distance of flow) from 9 to more than 70 hr. They were able to show that with slow development smaller and more discrete zones were obtainable, and also, with long slow runs in which the developer ran off the paper, many slow-moving amino acids could be effectively separated.

D. RADIAL METHOD

This method, described by Brown (127) and Rutter (797), and many others (739), has several modifications. Two of the simplest will be described, one composed from papers by Proom and Woiwod (750,751) and Saifer and Oreskes (800):

The method starts with a paper disk. This should not be creped, but smooth paper. The diameter will depend on the chamber used. For purposes of illustration assume an 11.5 cm. disk. One disk is creased to find

the exact center, and this is used for marking the others. The initial mixed zone is placed at the center of a disk of paper. When it has dried, two cuts are made, one on each side of and parallel with and equidistant from a radius of the disk to the zone. This gives a flap, or tab, of paper which is now bent downward to form a wick; the crease at which it is bent should pass through the center of the zone and be at right angles to the long edges of the tab. (See Fig. VII-5.)

The chromatographic apparatus consists of a large (14 cm.) culture or Petri dish, and the lid of a 9 cm. Petri dish (see Fig. VII-6). A large filter paper, damp with aqueous phase produced when making up developer, is placed in the bottom of the 14-cm. Petri dish, and on this is placed the inverted lid of the 9-cm. dish. Into this dish is introduced the developer, and the filter paper is placed on top with the wick centered and hanging well into the developer. The lid of the 14-cm. dish is now put over the assembly.

Modifications of this technique have been many. Methods of feeding the developer to the disk were described in the first section of this chapter.

Pollard and McOmie (739) make a sandwich of the filter paper disk between two glass plates, the lower of which has a hole drilled in it. The wick protrudes from this hole. The glass plates are clamped together or held by rubber bands, and the assembly rests on the Petri dish lid with the wick dipping in the developer. With a 10-cm. paper, development is sometimes complete in 15 min., and the glass plates protect the mobile phase from evaporation.

The working up of the chromatogram: drying, spraying, and measuring R_F values is as described above.

The method is simple, often very rapid, and capable of giving striking results. The velocity of development can be controlled by the width, or capacity of the wick, and very slow, or very fast runs can be made.

For purposes of comparing unknowns with reference compounds several initial zones may be placed on the same disk. The zones may be spotted on a circle concentric with the periphery of the disk, leaving a small space (0.5 cm.) between them (318), or one or more spots may be put on the concentric circle, developed with a given solvent, the disk dried, spotted at another place, developed and dried, and again spotted and developed, so that a record of the progress of multiple developments can be obtained on one disk (320). Alternatively, a row of three spots with short distances between them may be placed as an arc at the bend of the wick, two unknowns one at each terminus of the cuts, and a reference between. One of these modifications is shown in Fig. VII-5.

E. FRONTAL ANALYSIS

This is related to the old method of "capillary analysis," reviewed in detail by Weil (973). Essentially, a strip of paper dips into the mixture to be separated (there is no development step), and as the mixture rises up the strip the components that move most into the stationary phase (by whatever mechanism) are most retarded, so that the chromatogram eventually appears as a series of fronts. The liquids behind the fronts increase in complexity toward the trough of original mixture because some of each component that was less retarded is present with each substance that forms a front. Thus pure solvent, and a certain amount of one component plus solvent may be separated (Chapter II, Section IV).

Frontal analysis is important because it may occur with developers (as discussed above). The *developer*, being analyzed in this way, may abruptly change in composition at some region of the chromatogram.

Frontal analysis as a method was used by Wood and Strain in their analysis of rates of flow and distribution of liquid in filter paper (1022).

F. GRADIENT ELUTION ANALYSIS

Hais (362) has pointed out that in effect the frontal analysis that occurs in some developers may provide a gradient which could be used in a gradient analysis method. He has discussed the possibility also in connection with effects due to the presence of salts.

The effects of gradients were particularly marked in the paper chromatography of proteins (591). The spots of these substances were well rounded under conditions where the gradient in the developer moved the rear boundaries enough to prevent tailing. Amylases and diastases were best chromatographed with a developer of 50% ethanol, 1.5% in sodium chloride in a chamber the atmosphere of which was saturated with water. This atmosphere decreased the alcohol concentration in the mobile phase and affected the gradient along the paper. The salt, which at this low concentration increases the solubility of the protein, aids desorption at the rear of the zone and helps to prevent tailing.

Lederer (533) applied gradient elution to inorganic separation on paper. The upward-flow method was used. The initial developer, placed in the bottom of the chromatography jar, was stirred with a magnetic stirrer, and the gradient was produced by adding acid from a burette through a hole in the lid of the jar. The acid was introduced in definite amounts as the front of the developer passed marks previously made on the paper at 1-cm. intervals.

G. DISPLACEMENT ANALYSIS

M. Lederer (532) reported displacement analysis of rare earths on paper with solutions containing acetylacetone, acetic acid, and water, and in some cases butanol. He reported that the separations obtained on paper did not follow the same order of R values as in ion exchange columns, that is, the order was not that of the atomic weights.

XIV. FACTORS THAT INFLUENCE R_F VALUES

1. Analysis of the Problem

The R_F has two aspects. One is observational and operational: the ratio of the distance moved by the zone to that moved by the front of the developer. The other is theoretical: $R_F = A_M/(A_M + \alpha A_S)$. Hardly anyone attempts to measure the cross-sectional areas of the mobile and stationary phases (A_M and A_S). And it remains in doubt whether the α of the definition is the same as α measured in a separatory funnel or flask since, as Martin pointed out (612), the stationary phase may consist of imbibed cellulose (when the support is paper). So, the theoretical definition is chiefly useful as an intellectual scaffold upon which to hang an analysis of the R_F value such that the observed R_F values and their changes can be understood and manipulated. Such an analysis is particularly necessary because of apparent contradictions in the literature. Because of lack of knowledge in some areas we are still, as Hais put it (362), limited to morphological description instead of causative discussion. Many times the descriptions are opposed because some underlying unifying factor is not apparent. In their first paper Consden, Gordon, and Martin (191) called attention to the factors "paper, temperature, quantity of amino acid, extraneous substances, degree of saturation with water, supply of solvent, and distance between starting point and source of solvent" as influencing the R_F value. Zimmermann added length of path of development to these (1040).

In the analysis of R_F made in this section we examine the factors that influence R_F at two "levels," the bulk level and the molecular, but it must be emphasized that all these "separated" factors are contingent. *We are dealing here with a system*, and we break it down for purposes of discourse. The initial analysis is shown in Table VII-5. It has already been pointed out that, other things remaining constant, increase in A_M should increase R_F value; and increase in α should decrease R_F. The definition of α as (concentration in the stationary phase)/(concentration in the mobile phase) confirms that movement into the stationary phase (larger α) will decrease the R_F value.

TABLE VII-5

Factors that influence R_F

Through the system
(measurement, temperature)

- Bulk factors
(These are mechanical-physical properties of the system which exert their effects largely through their influence on A_M and A_S.)
See Table VII-6.
- Molecular factors
(These are the "chemical" properties, and operate largely through their influence on α, but also through effects on A_M and A_S.)
See Table VII-7.

TABLE VII-6

Bulk factors that operate through:

- Supporting medium (paper) (porosity and surface-bulk ratio; shape factors; homogeneity)
- Stationary phase (thickness and uniformity; changes with time)
- Mobile phase (thickness and uniformity; composition; changes with time)
- Solutes (zones) (position of application; amount applied; changes with time)

Stationary phase + Mobile phase:
Gradients
Shape-produced
Physical

TABLE VII-7

Molecular factors that operate in:

- Supporting medium
(Nature of surface and its modifications; structural properties; impurities; adsorptions
- Stationary phase
(Binding to support; polarity; composition)
- Mobile phase
(Polarity; composition)
- "Interface"
(Adsorptions; orientations; barriers)
- Solutes (zones)
(Amount applied; polarity; chemical structure)

Stationary phase + "Interface":
(Miscibility; effects of additives; effects of pH; changes with time: in composition; in miscibility; in concentration)

2. Measurement

Studies of the R_F are influenced by its reproducibility. Many careful experiments have indicated that when conditions are duplicated as closely as possible, R_F values can be reproduced within ±0.02 unit (43,800). When working with very complicated mixtures, such as might be obtained from natural products, it may be difficult to ensure such reproducibility as this. With a simple mixture and radial chromatography, Saifer and Oreskes (800) found the values given with a measure of their reproducibility in Table VII-8.

TABLE VII-8

Reproducibility of R_F Values[a]

Amino acids	Micrograms	No. of detns.	Range of values	Mean	Standard deviation
Glycine[b]	7.5	32	0.46–0.53	0.49	±0.023
Alanine[b]	8.9	32	0.58–0.62	0.60	±0.014
Valine[b]	11.7	32	0.74–0.77	0.76	±0.017
Glycine	7.5	16	0.45–0.49	0.48	±0.011
Alanine	8.9	16	0.59–0.62	0.61	±0.007
Valine	11.7	15	0.76–0.79	0.78	±0.000

Typical Set of Results for Calculation of R_F Values

Amino acids		R_F values (measured along radii)	R_F avg., calcd.
Glycine[b]	7.5	0.51, 0.47, 0.50, 0.49, 0.51, 0.51	0.50
Alanine[b]	8.9	0.61, 0.56, 0.59, 0.62, 0.59, 0.61	0.60
Valine[b]	11.7	0.78, 0.73, 0.75, 0.76, 0.75, 0.76	0.76

[a] From Saifer and Oreskes (800).
[b] All three amino acids chromatogrammed together.

3. Temperature

The effects of temperature were discussed in a section above. They are not all in the same direction for all solvent systems, but each system must be considered. For example, phenol becomes more miscible with water above room temperature; collidine becomes more miscible below room temperature. These effects will be discussed in detail below.

4. Bulk Factors in the Support

For bulk factors that influence R_F and for the physical properties of the system see Table VII-6. (We omit discussion of glass, quartz, nylon, and other kinds of "paper" as premature, though very likely the principles adduced here may, *mutatis mutandis*, be extended to these substances.)

Among the physical properties of the supporting medium, porosity

of paper is a major factor in controlling rate of flow of developer. In general, the larger the capillary pores, the faster the flow; thus the faster papers are also usually (but not always) thicker and softer, and the thinner, harder papers are usually the slower ones. But Whatman No. 120 is thick and yet slow because it is a dense paper (27). So porosity is the important feature, and the quickest way of testing velocity of flow is to test the paper. When flow rate is changed *without changing the paper*, there is a small but detectable increase in R_F with increase in velocity of developer (433). When *different papers* are compared, in general, the faster running papers produce higher R_F values (491,492) (see Table VII-9), but this is not an invariable rule. A "principle" such as "large (small) rate of development—large (small) R_F values" is too superficial to be used for generalizing.

TABLE VII-9

Comparison of Fast and Slow Papers[a]

Paper	S & S 589 Black Ribbon	S & S 604	W 4	S & S 507
"Collidine"				
Distance, cm.	39	41	42	39
Time, hr.	8	8	17	53
R_F				
Aspartic	0.13	0.09	0.10	0.18
Histidine	0.13	0.16	0.20	0.19
Proline	0.21	0.22	0.25	0.22
Valine	0.32	0.33	0.36	0.30
Phenylalanine	0.44	0.45	0.56	0.51
"Phenol"				
Distance, cm.	37	36	42	42
Time, hr.	5	7	14	48
R_F				
Aspartic	0.38	0.20	0.20	0.28
Glycine	0.53	0.48	0.46	0.40
Arginine	0.49	0.67	0.64	0.40
Hydroxyproline	0.75	0.72	0.72	0.67
Valine	0.83	0.84	0.80	0.78[b]
Phenylalanine	0.94	0.91	0.90	—[b]

"Collidine" (distilled from crude collidine; range 46°–63° at 10 mm. taken) was saturated with distilled water; "Phenol" consisted of 80% phenol, 20% water. S & S, Schleicher and Schuell; W, Whatman. Each group of amino acids was run as a mixture.

[a] From Kowhabany (491,492). In all cases descending development was used.

[b] Distortion of zone occurred.

It has been pointed out that, partly because of the geometry of the system (shape factors) (and because the liquid flows horizontally), the mass gradient in the developer in a radial chromatogram will in general

show a different profile from that in upward or downward development. If the ratio of A_M to A_S is different in the two methods, then the R_F values for the same substance would be different if everything else were constant. It has been suggested that a new R_F value, R_{FC} (a "circular R_F value" (760)) be defined (894). It appears that the R_F values for amino acids obtained by the radial method are higher than those obtained in upward or downward development.

Specially shaped papers have been used for particular purposes. For example, Lacourt, Sommereyns, and Wantier (512) used triangular-shaped papers for separating mixtures of ions where one was in large excess over the others. When the ion present in excess has a low R_F value, it is developed (as a streak across the paper) from the base toward the apex of the triangular piece of paper. The large amount of material which does not move far from the base has plenty of paper to be spread out on. The materials that are present in small amount, with higher R_F values, move toward the apex of the triangle, becoming concentrated into less wide and more readily visualized zones. With the excess material showing a high R_F, the original streak is placed near the apex of the triangle and development proceeds toward the base.

The effect of **length of run,** other things being equal, appears to be that with amino acids the R_F is lower for a short run and increases to a "steady-state" value (495), this effect being greater for the lower R_F substances and not very marked for higher R_F substances (800).

Inhomogeneity in the physical structure of the paper can show itself in irregularity of the advancing front of solvent or of a zone in the form of a strip across the paper. Consden, Gordon, and Martin (191) reported, however, that the effects of such irregularities on R_F were small, *ca.* $\pm 2\%$, for valine in phenol water on Whatman No. 1 paper, and could largely be eliminated by measuring from the center of gravity of the zone.

The differences between chromatograms run in the machine direction and across the machine direction of the paper, where they exist, seem largely to be differences in velocity of flow and in definition of zones and only rarely in R_F values (491).

Mixed zones, placed as spots close to the edge of a strip, may swerve and become distorted. However, zones may be extended from edge to edge of a strip and be developed quite regularly.

5. Bulk Properties of the Stationary Phase

(Reference to glass, synthetic fibers, chamois, etc., is omitted for lack of data.) The **thickness** of the supported phase enters the expression for R_F as A_S. If, other factors remaining constant, the cross-sectional area of the supported phase were to increase, relative to A_M, then R_F would be

decreased. Such a factor may operate in some cases where otherwise identical papers show all R_F values lowered. For example, Saifer and Oreskes reported (radial chromatography) that the presence of the water-rich phase (phenol isopropylalcohol water system) in the chamber resulted in a small decrease in R_F value for the faster running amino acids. The values in the presence of the water-rich phase were more variable and the zones were more irregular and diffuse; also the developer ran faster.

Schute (831) has analyzed the nature of the stationary phase as follows (856). There is a water of hydration of the cellulose fibers which, according to Hermans (386), may amount to about 7% at 20°C. and relative humidity 65%. There will be an imbibition phase, the water and other polar liquid imbibing the cellulose fibers in the amorphous regions to give what Schute likens (in agreement with Martin (612)) to an aqueous solution of a polyol, which may act as a stationary phase even when the developer is a single pure solvent. In addition, during conditioning some capillary condensation may occur, consisting of the capillary liquid that is bound by stronger forces. Then, on dipping the paper into the developer, the larger capillaries become filled by the rising liquid, while the liquid in the smaller capillaries, that gathered through capillary condensation may not flow, and that obtained by imbibition surely would not flow.

It was reported by Manegold and Solf (603) that a dried filter paper showed a specific weight (not including the empty space) of 1.57. Including the empty space it was 0.46. After the paper had stood 8 days in a desiccator over sulfuric acid its thickness was 1.42×10^{-2} cm., and the volume of empty space per milliliter was 0.71. After 8 days in a water-saturated atmosphere the thickness was 1.92×10^{-2} cm. and the volume of empty space per milliliter, 0.79. Swelling had thus increased the thickness and the fractional volume of empty space in the paper. Müller and Clegg (668) observed that the velocity of rise of water into filter paper increased with the time of exposure of the paper to a saturated atmosphere, reaching a maximum after about 30 min., though the paper continued to take up water for several days. This seems to indicate that swelling due to capillary condensation and possibly superficial imbibition changes the porosity more than does the slower imbibition of the fiber by water.

When the developer phase is a binary or more complex one, it is very probably the more polar part (often water) that is taken up preferentially by the cellulose. It seems extremely likely that this is the case because when a developer composed of an organic liquid containing water is used with amino acids the R_F values of the amino acids all decrease with decrease in water concentration of the developer and it must be that the lowered ability to dissolve amino acids due to the lowered water content of the developer is competing against a predominantly aqueous stationary

phase (495). This must be largely a solubility effect, and the effects on A_M and A_S must be incidental. It is known, however, that filter paper preferentially sorbs water from the collidine-saturated-with-water developer (495).

The **composition** of the stationary phase may also change with time through leaching by, or, in other cases, perhaps, through reactions with, the mobile phase. The former type of behavior was noticed by McFarren (581) when he observed that when papers alone were buffered (although reproducible R_F's of amino acids were obtained) a marked pH gradient was observed from the top to the bottom of the paper. Smith (864) observed the same behavior in partition columns buffered with phosphate and paper buffered with phthalate. He laid it to the leaching of acid from the upper parts of the column or paper by the mobile phase. Smith pointed out that a gradient such as that observed: less acid at the top of the paper and more acid below should give a progressively falling R_F value to basic substances and a progressively rising R_F value to acidic substances. (See gradient elution, above.)

Effects of **reactions** occurring in the phases will be taken up in the following section. Effects of *thickness* and *uniformity* of the stationary phase have already been discussed but the situation is unsatisfactory because actual information remains meager.

6. Bulk Properties of the Mobile Phase

These affect R_F through A_M. Any factor that changes the effective cross-sectional area of the mobile phase, for example, a change in composition that affects the surface tension, will affect the R_F, other things being equal. Such effects are, however, difficult to isolate and probably would be closely associated with changes in the stationary phase.

Thickness and **uniformity** have already been discussed, as well as the *frontal analysis* that may occur in the mobile phase. *Composition* and its effects on R_F will be left for Chapters XIII and XIV.

Waterlogging is a phenomenon that occurs when the paper becomes saturated with water. Consden, Gordon, and Martin (191) pointed out that it can have a deleterious effect by distorting the zones. Waterlogging can be caused by fall in temperature in chambers where the atmosphere is already saturated, thus depositing excess liquid on the sheet and at the same time causing a separation of the phases. Phenol saturated with water is sensitive to fall in temperature, and that is one of the reasons why undersaturated solutions are used. Waterlogging may be caused by the presence of salts in the paper or in the liquid in which the initial mixed zone is applied. The salts can "extract" water from

the atmosphere of the chamber and produce a waterlogged area (191,581). Waterlogging may also result from chemical reaction. Hanes and Isherwood (365) reported that when formic or other strong acid was used as a developer with primary or secondary alcohols, esterification occurred rapidly enough that the developer deposited water as it passed along the paper, producing waterlogging. This was avoided by using tertiary alcohols where the OH group is hindered toward esterification. When papers are impregnated with buffer salts, waterlogging can be prevented by buffering the developer also (581).

An interesting technique in which a section of the paper is purposely heavy-laden with the organic phase in order to provide a controlled distribution process of a special kind has been described by Allouf and Macheboeuf (11). The paper strip is dipped through a two-phase system in which the organic phase (they used isoamyl alcohol) forms a layer above the aqueous phase. A front representing the alcohol rises slowly (more slowly than the pure alcohol alone rises in filter paper). After this has risen to a certain height another front, an aqueous front (which also rises more slowly than pure water would rise in the paper), is seen to follow it, gradually overtaking it. As the aqueous passes the alcoholic front the latter ceases to rise, while the aqueous continues on. One then has a condition for differential countercurrent liquid–liquid distribution. The authors suggest that the paper imbibes alcohol, which rises, until the rising water front, traveling from the lower layer of the two-phase mixture, reaches the top of the alcohol phase. At this point it cuts off the source of supply of the alcohol from the paper. Continuing to rise the water expresses some alcohol from the interstices of the paper, thus allowing the alcohol front to extend itself somewhat, the limit being reached when the aqueous front just overtakes the alcoholic. The authors state that with their technique of a stationary organic phase R_F values were obtained for two pyrimidine bases which were different, and more conducive to a separation, than those obtained by conventional partition chromatography. The phenomenon, though not the technique, recalls as an aid to the picking of suitable systems, the studies of the ability of one liquid to displace another one which is sorbed (169, p. 107).

7. Bulk Relations between Stationary and Mobile Phases

When the developer is a single pure liquid such as water, there may be a stationary phase of imbibed cellulose and associated interacting film, as described above (612). It is difficult in such a system to differentiate between an absorptive and an adsorptive process. Schute (831) found that alkaloids showed lower R_F values (greater interaction with the stationary phase) when papers were not conditioned than when they were

conditioned. The presence of water on/in the paper may have decreased the strength of the sorption of the cellulose for the alkaloids. Certainly, in any case, the α that applies to a stationary phase is difficult to estimate. It must be expected that in this area of constrained supported phase, solute molecules will have activities altered in ways difficult to predict.

When the developer consists of completely miscible liquids, a stationary phase such as that visualized by Martin (612) is still possible and likely. The liquids being miscible will usually be alike (even if it is only in that they are small molecules). So if they are polar, both will probably imbibe the cellulose, leading to a phase such as that described above. If one is more polar than the other, e.g., acetic acid:ether or absolute alcohol:benzene, then it is most likely that the polar one of the pair will imbibe the cellulose to a greater extent and form the larger component of the stationary phase.

If the developer consists of a solution of liquids that are not completely miscible and are thus very much unlike, the tendency of the more polar to imbibe the cellulose will be even more effective in producing unlike stationary and mobile phases.

When the developer contains nonvolatile material and the stationary phase is essentially nonvolatile, as a propylene glycol:ligroin stationary phase and a ligroin developer containing dissolved propylene glycol (811), the stationary phase must be applied to the paper by impregnation, preliminary to chromatography, for the developer cannot furnish it in sufficient quantity.

In these cases, conditioning will almost always, it seems, tend to increase the difference between the stationary and mobile phases. The effect will be least for the pure developer; greatest for the developer of incompletely miscible liquids. But it is not possible to make a blanket statement about what conditioning will do to the R_F. The nature of the substance has to be taken into consideration, and also the developer. For example, with amino acids it is observed that conditioning may increase the R_F of some; sometimes very much. In a dry chamber the values for tryptophan and phenylalanine were 0.29 and 0.46 on paper buffered at pH 6.2 and developed with *o*-cresol saturated with water. When the chamber contained the water-rich layer on the bottom, the observed R_F values were 0.46 ± 0.07 and 0.64 ± 0.06 (581). Under the same conditions the R_F values of alanine and proline remained unaffected. Boman (99) observed a case in which conditioning the paper changed the R_F from a value considerably lower than 1 to 1 (see below). On the other hand, Schute reported that when antipyrine and pyramidon were chromatographed with butanol saturated with water R_F values of 0.82 and 0.87 were found. After 80 hours conditioning the R_F's were 0.74 and 0.76. The explanation of these effects must await the completion of this section on Factors.

Effects of shape-produced gradients have been considered above under shape factors.

8. Bulk Factors in Relation to Solutes

The position of application of the mixed zone, relative to the surface of the developer, has a marked effect on the R_F's of the components, as was pointed out by Consden, Gordon, and Martin (191). Studying the amino acid valine Kowkabany and Cassidy (495) found that the R_F decreased in a linear way with the distance that the initial spot was placed from the collidine–water developer, and that the effect was greatest when the mole fraction of collidine was the highest examined (Fig. VII-14). The lower the mole fraction of collidine, the lower is the R_F. This effect of location of the mixed zone was confirmed for radial development (800). This effect of the location of the spot of an amino acid was lessened when the paper was conditioned, though conditioning itself had no appreciable effect on R_F in the cases studied.

Zimmermann (1040) writes that the effect of the position of the spot on the R_F value of the amino acid is completely explained on the basis that the further the spot is placed from the developer surface the slower will be the velocity of the solvent when it reaches the spot. On the argument that slow velocity gives low R_F values this would explain the observed effects. However, it seems that the effects observed cannot be explained in this way, as will be shown in the discussion below. It should be pointed out, also, in this connection that with very low R_F substances—so low that multiple development is necessary—the position of the initial spot relative to the surface of the developer had no observable effect on the R_F value, is shown by French and Wild (298) and Jeanes and co-workers (448).

The **amount of material** applied in the initial zone may or may not affect the R_F values of the components. With amino acids, Consden, Gordon, and Martin found little or no effect (191); Kowkabany and Cassidy (495) found no effect over a range of 0.2 μl. to 3.0 μl. (applied in successive 0.2 μl. aliquots); Saifer and Oreskes (800) with a radial method found little or no effect in increasing the mixed zone from 10 to 25 μg. With enzyme preparations Giri and co-workers (319) observed that an increase in the concentration of the enzyme in the initial zone decreased the R_F.

It has been observed with ionizable substances that the **pH of the sample** may affect R_F as well as the diffuseness and other properties of the zone. An extensive study of amino acids from this point of view was made by Landua and co-workers (517). The more basic the solution in which glutamic acid was applied, the lower the R_F, for example, and the more basic the solution of arginine and histidine, the higher the R_F. But the

developer played a role also, and at some pH's there was a great deal of diffuseness, streaking and double zoning.

When much salt is present in the initial mixture, for example, of a protein hydrolyzate, it tends to produce waterlogging and immobilizing of amino acids. Consden, Gordon, and Martin (191) found that impregnating the paper with salt and conditioning it over the salt-containing developer eliminated the effect. But they abandoned this expedient because the ninhydrin colors then were slower in appearing, fainter, and redder than the usual ones, and substituted preliminary desalting of the hydrolyzates (192). Partridge (710) also found desalting necessary in the chromatography of sugars, because of the distortion and tailing of zones that was observed when inorganic matter was present.

Desalting has been achieved in many different ways, a discussion of which has been given by Block, Durrum, and Zweig (84). The methods can be classed as electrolytic, ion exchange, and solvent desalting (and there may be other techniques). Joseph (454) has used a device for electrolytic desalting of amino acids, peptides, and other substances. Platinum electrodes dipped into dilute hydrochloric acid inside glass cylinders, the ends of which were closed with cellophane. About 1 ml. mercury rested on the cellophane. These electrodes dipped into the solutions to be desalted. Anions reacted with the mercury to form insoluble precipitates; cations on discharge amalgamated, and eventually reacted with the dilute hydrochloric acid, releasing gas. The progress of the desalting could be followed by the evolution of the gas. Other electrolytic desalting devices have been published (20a,192). Apparatus is commercially available (see Appendix). Stein and Moore found that large losses of arginine and slight losses of other amino acids occurred during electrolytic desalting using the device of Consden, Gorden, and Martin (192).

Boulanger and Biserte (98) used Permutit 50 to demineralize, purify, and separate amino acids and related substances into two groups. Not fixed to the exchanger, they reported, were taurine, glucidic derivatives, and some polypeptides, whereas amino acids and derivatives and some oligopeptides were fixed. Brenner and Frey (109) passed the amino acid solutions through two exchange columns. Anions were picked up by a weak-base column (Amberlite IR4B) and cations by a weak-acid column (Amberlite IRC50). Neutral amino acids remained in solution. Piez and co-workers (734) removed amino acids from solution (leaving salt behind) with a lightly cross-linked strong-base anion exchanger. They used 4% cross-linked Nalcite SAR (60–100 mesh). The amino acids were eluted from the resin with hydrochloric acid, and the chloride was then removed in a batchwise operation by an 8% cross-linked resin in the bicarbonate form. They report complete recoveries within the limits of their analyses.

except that arginine is nearly completely lost, and lysine partly lost. Polypeptide and nonionizable substances are at least partly removed.

Boulanger and Biserte (96) desalt and defat plasma or serum by the following process. The sample is evaporated to dryness and extracted three times with redistilled acetone containing 1 ml. 10 *N* HCl per 100 ml. acetone. The extract is evaporated to dryness at 37°C. and the residue is taken up in a little distilled water. The aqueous, now desalted, solution is extracted with ether to remove fats. Amino acids remain in the aqueous layer.

Working with sugars, Malpress and Morrison (599) found ion exchange desalting slow and uncertain. They dried down their samples on a boiling-water bath, extracted with dry, redistilled pyridine, grinding the solid material into the pyridine, for 10 min. at 100°C. The pyridine was removed *in vacuo* at not over 40°C., and the sugar residue taken up in the minimum amount of water was concentrated for chromatography.

9. Molecular Factors. Chemical Properties of the System

See Table VII-7. The molecular factors that affect R_F are largely manifested in α. The ratio α represents the results of *competition between mobile and stationary phases for the solute* and may also reflect another competition: that between supporting medium (the cellulose) and the mobile phase for the components of the stationary phase. This latter competition may also be reflected in A_M and A_S, and has already been discussed.

10. Molecular Properties of the Paper

Filter paper is composed largely of the inert and insoluble α cellulose, but there are also present some aldehyde and acid groups. The aldehyde groups are the result of partial degradation of the cellulose and are determined as a "copper number," the number of grams of copper in the cuprous oxide resulting from the reduction of copper sulfate by 100 g. of paper fibers (913). A good filter paper is found to have a copper number of 0.4 or less. The acid groups probably result in part from oxidation, and in part are built into the fibers along with cations, as salt groups.

The effect of reducing groups in the paper is not entirely clear. An observation made by Turner (949) and reported in Ott (702) is possibly suggestive. Conductivity water was allowed to rise up a highly purified cotton fabric. A brown line was formed at the front where the water evaporated (the highest point of rise). It could be reformed with undiminished intensity at lower levels on the same fabric by raising the fabric, the lower part still remaining in the water. The brown material was soluble in alcohol and showed reducing power. This kind of reaction may

be partly responsible for the "brown front" observed in some chromatograms. However, it should not affect R_F, since the brown front runs faster than most substances that are separated, except in phenol (191). It has been found that the same R_F values are obtained for amino acids using paper from the brown front with the stains present, as paper from the same sheet above the brown front, where the paper had been washed, and below, where the developer had not yet reached. The brown front was produced with a collidine–water developer in downward flow (495). The heavy brown front obtained with poor grades of phenol (226) is laid to decomposition of developer catalyzed by traces of copper ion in the paper (191).

Two classes of *impurities* can be distinguished in connection with the paper used in chromatography. The first comprises substances continuously formed, such as that mentioned above, or hydrolytic products formed on long treatment of the paper with acid (672). This class seems to be not very important in ordinary work. The other class comprises impurities in the paper which can be removed once and for all from the strip—the "extraneous substances."

The presence in the paper of extraneous substances may affect R_F. If the impurities are present in very small amount they may only affect the definition of spots. Thus pink fronts on amino acid spots which appear after reaction with ninhydrin were observed and laid to the presence of copper ion in the paper (191). These did not seem to affect R_F and could be obviated by the use of specially purified paper (494), prewashed paper, or complexing agents in the developer (191). In this class belong also, perhaps, the peptide-like contaminant reported by Wynn (1025), the dark zone-producing impurities (696), and probably many others. (Not included here, of course, are those impurities introduced with the developer or solutes.) These impurities may affect R_F, but usually cause trouble by making it difficult to recognize the center of density of the zone or by producing the above-mentioned artifacts. However, larger amounts of noncellulosic material present in the paper must obviously be able to affect R_F through their effects on A_S, A_L, and even α. Thus it was found (494) that a paper which is melamine-treated for high wet strength shows a greatly retarded aspartic acid spot (phenol–water developer) compared with Whatman No. 1 paper, and greatly speeded histidine spot (butanol–ammonia). Interesting possibilities for improved separations lie in the use of such papers. The "Ghost" spots observed by Kennedy and Barker (467) and eliminated by an oxalic acid wash may not be caused by impurities (see below).

The inorganic cations in paper are thought to be present largely held by carboxylic acid groups (509). The inorganic material does affect adsorp-

tion by the paper (487) and may play an important role in chromatography through ion exchange. If present in relatively large amounts, as in some industrial filter papers, it may affect R_F.

Inorganic substances are, of course, purposely put into the paper for various reasons: to prevent water-logging; to act as buffers; or to act as actual adsorbents, thus complicating the nature of the "supporting medium." This has been described above.

Paper, being made largely of chemically unmodified cellulose, is chemically asymmetric. It has been reported (490) that the *d*- and *l*- forms of the acidic amino acids glutamic acid and tyrosine give slightly different R_F values on paper, and that racemic tyrosine-3-sulfonic acid can be completely resolved on paper. Optically inactive developers as well as developers containing *d*- or *l*-methyl (β-phenylisopropyl)-amine were used. The effects were laid to the asymmetric nature of cellulose; however, in most cases the effects are so small as to suggest that the paper is not adsorptively involved with the solute (671).

Finally under the heading of the molecular properties of the supporting medium, a rather interesting phenomenon (with a very large bibliography) needs mention. This has to do with the charge which filter paper may acquire (170), such that under given conditions (the application was in this case in capillary analysis) negatively charged colloids would rise as high as the solvent front, while positive colloids would not rise, or rise very little. (The positively charged colloids could be made to rise under special conditions.) The literature is to some extent reviewed by Garner (311). The field of possible application is broadened, too, by consideration of the observation of Friedberger (301) (for example) that Gram-positive bacteria (the term positive does not refer to charge, here, but to staining properties) are *generally* held back more than the Gram-negative by filter paper. He found that if a capillary tube containing finely divided filter paper were dipped into a fluid and the liquid allowed to rise above the filter-paper column one kind of bacterium might predominate in the liquid. (He was attempting to analyze feces for typhoid bacteria, and these preponderated in the liquid above the paper column.) His results were confirmed by Hofmann (412), who showed that the method was very sensitive to pH. One may visualize, from reports of these kinds, the extension of paper chromatography into fields of the separation of colloidal materials (besides viruses) and into bacteriology and ore flotation analysis.

11. Molecular Properties of the Stationary Phase

The paper surface is hydrophilic and is laden with hydroxyl groups from the glucose residues, whether these are bonded to each other or not.

it will tend to interact more with more polar substances. The bulk phenomena described under the headings of composition changes in the mobile and stationary phases, frontal analysis, and waterlogging are all explainable on this basis. If the stationary phase is firmly bound to the paper, it becomes less possible for adsorptive interactions to occur. This has been suggested by Schute (831) (see below) to explain some effects of conditioning.

12. Molecular Properties of the Mobile Phase

These properties are discussed in Chapters XIII and XIV and will not be treated further except in the discussion below.

13. Molecular Properties of the "Interface"

If the developer is a pure liquid, like water, then very likely the stationary phase of imbibed cellulose and associated atmosphere of loosely held liquid shades off into the mobile phase, and there is no interface between the two. An interface is a boundary between adjacent phases at which some property, constant in one phase, undergoes an abrupt change in passing to the other phase. Such an abrupt change can be present only between immiscible liquids. It is very likely present in the system reported by Strain (891) (see Chapter XI, Section IV).

14. Physical Properties of the Interface

No appreciable effect on R_F should be present. Some effect may be present on the definition of the zones, in that the "stationary film" which exists at the boundary of a moving fluid (430) does set up a barrier to diffusion of solutes, and hence some hindrance to attainment of equilibrium; but how closely the processes in paper chromatography approach an equilibrium is an open question. Boyd, Adamson, and Myers (100) found that diffusion through the stationary film might become an important rate-controlling factor in the ion exchange distribution in sufficiently dilute solution (*ca.* 0.001 molar). Presumably similar limitations may apply in paper chromatography where except for differences in charge density the supported phase may be much like the nonmobile exchange-resin surface.

Concerning the *system of mobile and stationary phases*, most discussion is reserved for Chapters XIII and XIV. Consden, Gordon, and Martin (191) pointed out in a general way the effects of pH. Thus an alkaline pH decreased the R_F of the acids aspartic and glutamic by making them more soluble in the stationary phase; and an acid pH operated in the same way on the basic amino acids. There has been some discussion of this under buffers and gradient analysis, above.

McFarren observed that different buffers of the same pH may cause differences in R_F of amino acids, and Hais and co-workers (362) call attention to the possible complexing of borate with hydroxyl groups. **Additives** are intentionally put into developers in chromatography of inorganic ions (739). For example, normal butyl alcohol saturated with 0.1*N* nitric acid and containing 0.5% (w/v) of benzoyl acetone complexing agent is a good general developer for cations. Some developers are "complexing" agents in their own rights, and do not need additives. Thus a copper chloride zone developed with *n*-butanol saturated with 3*N* hydrochloric acid shows an R_F value of 0.19. Developed with collidine the R_F value is 0.76.

The **effect of temperature** depend on the composition of the developer and on the nature of the solutes. For example, Nicholas and Rimington (689) found, in the chromatography of free porphyrins with a lutidine–water system that temperature change between 5° and 21°C. had little effect. The saturated solution of water in lutidine at 21°C. contains 40% water (v/v), and gave the same R_F values as a 40% solution at 5°C., at which temperature lutidine and water are completely miscible (they are miscible at any temperature below 17°C.). Kowkabany and Cassidy (495) found that at higher concentrations of water in a collidine–lutidine developer (35% to 39%, w/w) there was a small decrease in R_F value with increase in temperature from *ca.* 4° to *ca.* 25°C. with some amino acids (glycine, valine, and proline) but there was little effect with others (phenylalanine and aspartic acid). There was no appreciable effect when the water content of the developer was 30% (w/w) or less. Saifer and Oreskes (800) found that increase in temperature from 7° to 37°C. increased the R_F values of glycine, alanine, and valine in a phenol–isopropyl alcohol–water developer. These are only a few of the many results that have been reported, and which are referred to in the above publications.

It is the general observation, but not without exception (495), that in using developers composed of partially miscible liquids, changes in ratio of the components will change the R_F values but not the relative *order* of the zones.

15. Molecular Properties of the Solutes

The molecular properties of the solutes influence R_F largely through α. This is discussed at length in Chapter XIII.

XV. FACTORS THAT INFLUENCE THE DEFINITION OF ZONES

One of the requirements of a good chromatogram is that the zones be well defined and circular or slightly elliptical. In special cases deviations from the second of these requirements may be considered desirable; but

good definition is a *sine qua non*. But the working chromatographer, using new systems, examining unknown mixtures, is continually beset by diffuseness of zones, poor reproducibility, multiple zoning, streaking, flattening, swerving and other distortions, loss of zone material, and much more. The reasons for these troubles are seldom exhaustively investigated; the worker using paper chromatography as a tool is probably only too happy to solve his particular problem and get on to more interesting things. For this reason this section is inconclusive on very many points.

Perhaps one use it will have is to encourage the practicing chromatographer on two counts: where difficulties are concerned, he has much company; where causes for the difficulties are looked for systematically, the prognosis for cure is good.

Because of the inclusiveness of this category and the lack of information about the detailed causes of many of the phenomena dealt with, the classification scheme used in this section is more arbitrary than that used in the previous one. Table VII-10 shows the classification scheme to be used here.

TABLE VII-10

Factors that influence the definitions of zones

Factors related to diffusion (diffuseness; effects of flow)	Factors related to R_F (reproducibility of R_F; constancy of order; problem of proof of purity)	Factors producing zone irregularities (streaking or tailing; forward elongation; flattening; V-formation; swerving; smudging)	Factors producing artifacts (multiple zone; impurities; zone sharpness)	Factors producing losses (oxidation; heat; light; acid; base)

1. Factors Related to Diffusion

The chromatographer always has to contend with diffusion. In paper chromatography the attempt is made to localize different substances in sharply defined zones which are as concentrated as possible. Diffusion works against this localization of matter by its tendency to equalize concentrations in the system. Sharp definition of the initial zone is the first move by which the chromatographer hopes to minimize diffusion effects (110,292). The moment that developer passes over this zone (if not before, during the conditioning period) diffusion begins to blur its edges. Diffusion being a rate process, controlled by a concentration difference, it is ap-

parent that the chromatographer has two variables, time and concentration, which he can manipulate in dealing with this diffusion problem. But there is another, linked diffusion process to be considered. That is the diffusion upon which the distribution between phases rests. This sets limits to the other.

The chromatographer could minimize the effects of one of the diffusion processes by making the zones dilute and by running the chromatogram rapidly. The zones are already too dilute for the analyst who wishes to separate a fair amount of material. The process cannot be speeded too much (by the use of fast papers and developers) because of the second diffusion process which controls the rate of transfer between phases. Yet the developer has to run a certain distance (period of time) in order to separate the zones, and the larger and more numerous the zones and the closer the R_F's, the longer the development needed. An approach to shortening the period of development for an analysis was made by Rockland and Dunn (787) by using very small zones which did not need to travel far to become separated (assuming a reasonable difference in R_F). The distance one can go in this direction is obviously limited by the acuity of the method of recognizing the zones of substance.

Fortunately, the problem is not serious in practice. That this is so could be explained if the stationary phase were fairly well divided so that diffusion *in it* for any distance would be difficult. This could be visualized for a mat of fibers each sheathed in stationary phase films which joined where the fibers crossed or came into contact. Also the "stress" on the zone imposed by the moving phase probably subdues lateral diffusion.

Diffusion does cause difficulty if the developer is allowed to accumulate at the bottom of the paper in a downward run (191,452) or at the top in the upward method (288). It is noticeable if papers are left in the vapors of the chamber for several days after a chromatogram has been stopped (938).

2. Diffuseness

Under carefully controlled conditions, it was found that faster flow increased the size and diffuseness of spots (433), and this seems to be a general observation, for in a study of many filter papers, with amino acids, it was usually the fastest ones that gave the most diffuse, swerved and smeared spots. However, some very slow papers, like Whatman 5, and Schleicher and Schuell 576 gave diffuse spots with phenol developer (491).

3. Reproducibility

Reproducibility of R_F values was discussed above. In some cases the R_F is quite sensitive to small changes in the stationary phase. For ex-

ample, Sakal and Merrill (801) found that when corticosteroids were developed with a xylene:absolute methanol (225:75, v/v) developer the R_F was very sensitive to whether the paper had been dried beforehand or not. The R_F values with undried paper were always lower. The values with dried paper were more reproducible. The lowering of R_F reflects the increased polarity of the stationary phase due to the presence of the water, and the poor reproducibility very likely the variableness of the water content of different papers.

Although, in general, the **order of zones** of closely related substances in a chromatogram does not change with change in the ratio of components of the solvent, or even with change in the kind of solvent (see discussion in a previous section), such changes have been observed, and they can be confusing. They may be related to the kinds of intramolecular effects which cause the values of properties of the first few members of an homologous series to be "out of line" with those of the higher members of the series. With less closely related substances, changes in order of zones are common. In their classic paper, Consden, Gordon, and Martin (191) pointed this out in connection with amino acids. Thus acid in the developer had little effect on the neutral amino acids but slowed the basic ones and speeded the acid ones. Base had the effect of speeding the basic and slowing the acid ones. An example of this sort is recorded by McFarren (581). Run separately in *m*-cresol–saturated pH 9 buffer on paper impregnated with pH 9 buffer, tyrosine showed an R_F of 0.29; histidine, of 0.35. When mixed and run together, the R_F values were tyrosine, 0.26 and histidine, 0.14. The change was laid to the acid used in bringing tyrosine into solution. This was sufficient to overcome the buffer and lower both R_F values, but particularly that of the histidine. Effects such as this can change the order of zones in a chromatogram. Indeed, this ability to change the position of a zone is utilized as a means of recognizing substances by making apparent the presence of specific, chemically reactive groups.

For example, Dent (226) recommends placing a beaker of 50% (v/v) acetic acid in the cabinet during a phenol run to slow up basic substances like glucosamine. These can be recognized by seeing which spots are slowed compared with a phenol run without the acid. Concentrated HCl–water (1:1 v/v) may be used but will also slow some monoaminomonocarboxylic acids. As another example, sulfur-containing amino acids can be recognized by comparing an ordinary run with one in which hydrogen peroxide has been applied (with a trace of ammonium molybdate catalyst) to the initial spot or to the initial mixture. The sulfur-containing amino acids are oxidized, and yield lower R_F's in phenol.

The question of reproducibility of R_F's and reliability in the order of zones has implications for *tests of purity*, *homogeneity*, and *identity*. The most

acute tests of homogeneity or of purity involve an attempt to separate the substance into fractions, *combined* with a method of comparison of the possible fractions (165). The first part of the test is best achieved through a differential countercurrent separation method, such as chromatography. The second part then follows as a consequence of the method, since nonidentical substances with different R or R_F values are shown up. Since, however, the method may at times yield double zones with single substances it is important not to lay too much stress on a single chromatogram as a test of purity (or identity). For a test of identity the two substances to be compared should be run parallel to each other (see ref. 705), possibly also with a mixture of the two on the same strip. For confirmation the developer system should be changed, whereupon the conclusions from the test should remain the same. If double zoning is suspected, the two zones should be rechromatogrammed under the same conditions as before, when, if double zoning occurred each should split into two zones, and the behavior of each should be like that of the other. Double and multiple zoning is discussed in the section on artifacts, and the matter of testing purity is specially discussed in Chapter VIII, Section X.

4. Irregularities in Shape

Irregularities in the shapes of zones are among the less well-understood plagues of the chromatographer. The best zones show a fairly sharply defined outline and are circular or somewhat oval. That is to say, the length of the zone (in the direction of movement) is about the same as its width (at right angles to the direction of movement). The distortions observed may comprise streaking or tailing, in which the zone is elongated in the direction of movement, the tail stretching back and fading out in the direction of the starting line; elongation ahead of the zone; flattening, so that the zone becomes shaped like a crescent, a heart, or an arrowhead, a smeared longitudinal straight or wavy band; a smudge of uncertain shape with fuzzy edges; etc., *ad nauseam*.

One cause of streaking or tailing is overloading the system. In column chromatography the more material that is put on the column, the deeper the zone formed. This applies also to paper. The capacity of a given volume of mobile and nonmobile phases (and the associated paper surface) is often severely limited. If solute is piled up in an initial zone so that the capacity of the phases which would cover the area of that zone during development is exceeded, then the passing phase(s) may become locally saturated while solute still remains behind as the initial zone. Then, developer will continue to carry material from this zone until the amount remaining falls to the value at which the rear boundary of the zone begins to move. An elongated zone is then produced. The cure is to use less

material in the initial zone or to increase the capacity of the system. Streaking may also occur if the developer moves too rapidly (see above), so that the capacity of the developer is not utilized to the full (or nearly to the full, as is the usual correct condition) because each element of liquid passes over the zone too rapidly to pick up as much solute as it could. Thus efficient transportation of solute is hindered by rapid flow. One cure may be to use a slower paper; another, to feed the developer more slowly to the paper.

Another cause of streaking arises when the composition of the substance in the zone changes during the run. This may happen as the result of a chemical reaction with the components of the system: reactions such as a change in degree of ionization, of esterification, of isomerization. For example, change in degree of ionization can occur when the substance passes through pH gradient along the paper, and has been observed frequently with acidic and basic amino acids. The ratio of virtually unionized to salt form may be changed: if more salt form is produced it lags behind in the nonmobile, more aqueous layer, thus producing streaking; if more unionized form is produced, the R_F is increased and it runs ahead of the main zone, thus producing forward elongation (517,581). The cure is sometimes achieved by buffering the paper and the developer; at other times by adjusting the pH of the mixture which is applied to the paper (191,226,581). Many examples can be derived from the figures given by Landua and co-workers (517).

Ionization of acids, for example, may cause streaking, formation of "comets," as was observed by Lugg and Overell (571). The ionized form tends to remain in the nonmobile, aqueous phase, while the nonionized form tends to the mobile. This leads to severe streaking with strong acids such as the dibasic and hydroxy acids. It can be remedied by suppressing the ionization of these acids by adding a stronger acid (such as formic) to the developer. This not only prevents streaking, except in the case of the very strong oxalic acid, but also increases the R_F's of the acids (571). The theory of preventing this kind of streaking is dealt with in connection with the use of gradients, and in Chapter XIV.

The tailing due to ionization is a special case of tailing due to inconstancy of α. If the distribution ratio of the substance in all its forms is a constant over the concentration range of zero to that in the zone, the zone should move without change in shape, other things being equal. But if the distribution ratio is not constant, the shape of the zone is likely to change with development. Thus if the total concentration of substance in the water phase, divided by that in the organic phase, becomes larger with dilution (as might occur, for example, if ionization were not "complete" at the dilution of the initial zone, and the extent increases with

further dilution), then tailing would occur. This is because as material is transported from the zone by developer and the zone becomes more dilute, the ratio of material passing into the mobile phase becomes progressively more unfavorable, and transport less efficient. This is likely to be a contributing factor in the tailing of acidic and basic amino acids, and other ionizable substances. It occurs, in principle, for the same reason that zones tail in conventional chromatography. A cure is to use a sufficiently dilute zone that the distribution ratio remains constant with composition; another, to change the solvent system to one in which the distribution ratio remains constant; still another, to swamp out the effect as one might in the case where the tailing is caused by adsorption to the supporting phase.

If the tailing is due to adsorption of solute to the surface of the supporting medium, then the cure may be to add to the developer some substance which is still more strongly adsorbed and which can displace the zone-forming substance into the mobile phase (99,616).

Jermyn and Isherwood (452) report tailing of zones of sugars that were chromatographed with certain solvent mixtures (ethyl acetate, water, dioxane). The sugar locally "increased the mutual solubility" of the phases, which caused the zones to tail.

Sometimes an elongation is observed at the rear of the zone which is due to the presence of impurities in the paper. Such "shadows" behind the zone have been reported to be caused, in the chromatography of the phosphoric esters, probably by heavy metal contaminant. They could sometimes be eliminated by prewashing with 8-hydroxyquinoline solution, or in cases where this failed, by introducing a small amount of hydrogen sulfide into the chromatography chamber (365).

Sometimes the zone is elongated ahead of the main body of substance. This phenomenon was observed by Consden *et al.* with amino acids (191). When the finished chromatograms were sprayed with ninhydrin reagent a "pink front" or "beard" was found ahead of the main zone. Tests indicated that this was most likely caused by copper ions occurring as impurity in the paper. These formed salts with the amino acids which showed slightly higher R_F values than the parent acids, thus running just ahead of them. The remedy was effectively to remove the interfering ion, either by complexing it or precipitating it *in situ*; or by removing it from the paper by preliminary washing. A similar appearance, of a less intense zone running slightly ahead of the main zone, called "heading" by McFarren (581), may be the result of pH changes or changes in ionization, and may be remedied by the means listed in those connections. Diffuse front and sharp rear boundaries were observed by Spaeth and Rosenblatt (871) in column partition chromatography of anthocyanidins.

Evidently (to sum up the conditions leading to "tailing" and "running ahead" phenomena *caused by changes in nature* of the material of the zone) a zone which shows tailing not due to overloading gives evidence that during the development a slow-running species is produced from the original zone substance. This species is more hydrophilic in the conventional method of paper chromatography and more hydrophobic in the reversed phase methods. If the zone shows a forward trail, then a faster running species is produced.

To present one further example, Partridge (710) observed that on chromatography of a 7:3 mixture of glucuronic acid:glucurone two well-separated zones could be seen. The acid showed the lower R_F; the lactone the higher, as would be expected. Sometimes there was a forward trail from the acid spot. When the crystalline lactone was developed in acid developers it gave a single circular zone. When developed with basic developers the zone was often accompanied by a tail indicating conversion to the ionized form (opening of the lactone) during development. In the presence of a sufficiently basic developer the lactone zone was entirely absent, even though pure crystalline lactone was the component of the original spot, and instead, a zone of glucuronic acid (salt) appeared accompanied by a forward trail. Galacturonic acid lactonized with difficulty and accordingly on chromatography gave a well-defined spot with, occasionally, only a short forward trail. Partridge's analysis given above also held in other instances.

There are other causes of tailing and forward elongation. If the distribution coefficient α for the pure substance is not constant, and the distribution curve takes the shape B, Fig. IV-1, which is so typical of adsorptive interactions, then the zone will tend to tail unless the concentration is so low as to be in the nearly linear section of the isotherm. If the isotherm takes the shape C, then running ahead, or forward elongation, is to be expected, for the more dilute the zone, the more it will tend (relatively) to the mobile phase, and so the more effective its transportation.

Another mechanism of streaking has been suggested by Boman (99). If the substance is strongly adsorbed, or tightly held by a low-capacity stationary phase, the zone material, leached from the initial spot, is immediately immobilized and a long trail is drawn out from the spot. Thus albumin, placed on paper and developed with ethanol: phosphate buffer (pH 5.2) (3:7)), streaked badly. The albumin at this pH is strongly adsorbed. The effect is much less at pH 7.7, when both the albumin and the paper are negatively charged. But even here there is evidence of some adsorption, particularly when the paper is not conditioned. When the paper is conditioned for 8 hr. before the run, the albumin is so weakly held that it runs with the front of the developer.

If the substances dissolve slowly, there will be a tendency to tailing, or streaking, as the material leaches from the zone (831). This is the time analog of overloading.

Flattening of zones, a process in which they tend to become lens-shaped, is usually observed when the R_F value is very high: near 1. It can readily be visualized as a result of the turbulent motion of the liquid near the front of the developer as it rises in the paper (170). This seems to cause lateral motion and mixing of liquid in among the paper fibers, and would affect substances with R_F of almost 1. Flattening is reported to accompany repeated development (448). It affected substances with high R_F values and was accentuated by the repeated development. The mechanism suggested is that the lower edge of the zone, for substances with high R_F, starts to move before the developer reaches the upper edge of the zone, thus crowding together the two edges. The frontal analysis of the developer suggested elsewhere may play a role here. Hais (362) suggests that displacement may be involved, which carries this same implication. He says, also, that transverse spots, shorter than those originally applied but of the same dimension transversely, are provided especially by slow papers. Near the front he suggests that they may be explained by a drying out of the mobile phase. This might operate also with the mechanism suggested by Jeanes and co-workers. The flattening was found beneficial to some separations. To prevent it one would have to change the solvent system so as to lower R_F, or perhaps to use a slower paper.

Swerving of zones is sometimes encountered with little evidence of the cause for it. It seems to be associated sometimes with having the zone too close to the edge of the paper, when it seems to swerve over towards the edge. On the other hand, chromatograms on strips or sheets are run successfully with a zone extending from edge to edge across the sheet, with quite regular fronts. Swerving and other irregularities are sometimes laid to defects in the papers. It is probably a good routine measure to hold papers up before a light and inspect them for defects such as thin places and inclusions. Waterlogging may cause distortion of zones (191).

Sometimes "*ghost*" *zones* remain on or near the starting line in the region of the initial spot. Hanes and Isherwood (365) reported such ghost spots in the chromatography of phosphoric esters. They found that these could be eliminated by a preliminary wash with dilute hydrochloric acid and could be reproduced in severe form by treating the acid-washed paper with calcium or magnesium acetates to the original ash content.

Another possible cause of these "ghost" spots or "shadows" is proposed by Schute (831). Suppose that the paper has not been properly conditioned, and the mixture to be separated is put on the paper using a liquid that imbibes the paper. Then some of the mixture may pass into the

cellulose with the imbibing fluid and become inaccessible to development. He gives an example: azobenzene is placed on paper in methanol, and then, after the methanol has evaporated is chromatographed with hexane. Under these conditions a part of the azobenzene remains at the starting point—a "ghost" spot. Again, he says that when alkaloids in chloroform are put on the paper and developed with nonimbibing developers (such as hexane), such spots will be found at the starting line. The mechanism proposed is that the evaporating chloroform causes local cooling of the paper and consequent condensation of water on the spot. The water imbibes the cellulose and carries some alkaloid in, rendering it inaccessible.

Still another possible cause for these spots and shadows in the position of the initial zone may be surmised. In the mixtures applied to chromatograms there may be present peptized colloidal materials, held in suspension because of charge. Paper, which normally takes on a negative charge in aqueous solution, would flocculate positive colloidal materials, and these would likely be precipitated irreversibly at the starting line, giving a residue, a "ghost spot," or shadow. The large literature that has accumulated on the behavior of paper due to its charge may be entered through the references already given on that subject.

Another mechanism has been reported by Heyns and co-workers (397), who found that glutamic acid hydrochloride developed with butanol:acetic acid might give three spots on the chromatogram: one, glutamic acid; another the butyl γ-glutamate ester, R_F 0.62 formed at an early stage in development because of HCl present; and a cellulose ester of glutamic acid that remained at the point of origin.

Crescent or **V-shaped** or **heart-shaped zones** are frequently encountered. Schute (831) has suggested that these may sometimes arise in the following way. As the developer moves up to the initial spots and encounters them, there may be local changes in the surface tension or in the viscosity of the developer at these spots, or some of the pores of the paper may be blocked by the substance. Such interferences would disturb the linearity of the front so that developer would move faster or slower through the zone than on either side of it. The front would consequently be distorted, and bent in the direction of retardation or acceleration of the fluid as shown in Fig. VII-16. Such a mechanism might be expected to operate so that the faster moving zones might be most affected, and may account for the crescent zones that are illustrated by Paecht and Katchalsky (707).

5. Stains

Sometimes there are observed accumulations of substance (colored, or fluorescent) near the front of the developer, with streaks or lobes

("tongues") extending back from the front. Developers such as phenols and collidine, which are sensitive to oxidation, light, and metal catalysis may show these phenomena (191,362). Hais (362) reports such phenomena in connection with the analysis of extracts from lipoidal materials such as milk and blood. These could not be removed without at the same time removing some of the lipophilic materials being examined. Kowkabany and Cassidy (495) found such discoloration occurring as a brown area at the solvent front with a collidine developer. A sheet of paper with a broad, brown front developed in the machine direction was dried. Paper strips were cut from this sheet in the machine direction: one set from the

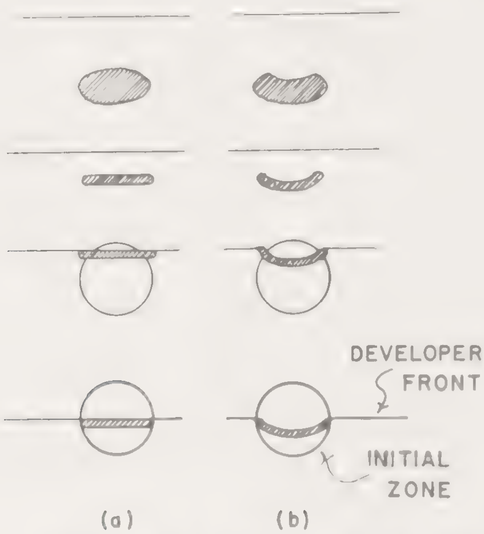


Fig. VII-16. Possible mechanism of curvature of spots. Four successive stages in the development of spots. Four successive stages in the development of a zone, showing the movement of substance normal, in (*a*) and disturbed, in (*b*). The disturbance in (*b*) might be caused, for example, by a clogging of the pores of the paper in the more concentrated central parts of the zone, with consequent local slowing of the front. (After Schute (831).)

discolored region; one from well behind the stains, where the paper had been washed by the developer; and one from well in front of the stains, where the developer had not yet passed. Valine was run on all these strips, and there was no evidence that the discoloration, or washing it out of the paper, affected the R_F value of the valine. The brown fronts and other discolorations, though giving a displeasing appearance to the chromatogram, do not necessarily cause difficulties except as they may obscure fast-running zones.

Zones are sometimes observed *to change in shape* during development. Boman (99) reported that with a (3:7) ethanol:phosphate buffer de-

veloper zones of albumin which started out oval in shape behaved as though they were taken up by a "front" running behind the front of the developer. This invisible second front seemed to displace the zones so that they were flattened and compressed. Evidently the developer underwent frontal analysis, and the zones were being developed in a gradient (see above).

Multiple banded spots (365), *diffuse dendritic forms* (362), and *other distortions*, are observed for which the causes are not clear.

6. Artifacts

The appearance of artifacts, though not very common, needs attention because of its implications for tests of homogeneity and tests of identity.

"Ghost" spots at the point of origin of the chromatogram have been discussed. In work with acids, the permanent acid background shown by some papers may cause trouble; and in investigations of amino acids and peptides, particularly where traces are of importance, the presence of ninhydrin-reacting substances may cause difficulty. Wynn (1025) found peptides to be present in many filter papers. He recommended washing the papers for 48 hr. with water.

Double and **multiple zones** have occasionally been observed to be produced by single substances. These seem to occur under more than one kind of circumstance. Substances which are pH-sensitive may show such behavior, especially where the zone moves in a developer that is undergoing frontal analysis. Aronoff (18) reported that lysine might give streaks with more than one center of gravity when applied at certain pH's and developed with phenol. He ascribed this to the presence of several ionic species with different distribution behavior. In Boman's observations with albumin (99) double zones appeared when the albumin was absorbed too strongly to be entirely carried by the advancing front of developer, and so was only partially removed from the initial zone, with a streak running back to the zone. Then, as the gradient in the developer (due to frontal analysis) reached a certain level at some distance behind the front (R_F value about 0.8), the developer was able to displace the residue of the albumin. This produced a zone which was connected to the leading zone by a diffuse trail. The second zone did eventually catch up with the first one, to form a single compact zone, if the distance of development was great enough. These kinds of double zoning, including those reported and discussed by Schroeder (826), Smith (864), and Ovenston (705) (Chapter VI, Section VII) all seem to be basically due to a gradient of pH and/or concentration in the developer, produced in some cases by frontal analyses of it during the development process; or by temperature change; or by changing developers.

Another kind of double zoning may occur when the substance undergoes changes during the chromatography, as in the case of glucuronic acid and its lactone, referred to above (710). The formation of unanticipated reaction products falls also in this category. For example, Bayly and co-workers (54) reported that single sugars might give more than one spot on paper. This turned out to be the result of a reaction (55). When the sugar solution was evaporated in the presence of ammonium acetate, a glycosyl amine was formed, and the chromatogram duly reported the presence of this new substance.

The apparent sharpness of a zone may mislead at times. It is to be expected that the apparent size of a zone will be to some extent dependent on the indicator used to reveal it. For example, Dent (226) has pointed out that although the Sakaguchi reaction may be used on paper to characterize arginine, it is of limited value because of lack of sensitivity (compared with ninhydrin). Thus on a chromatogram where arginine may be present, but undetectable to the Sakaguchi test, it might still show up to a ninhydrin test. In general it should be taken as most likely that all "zones" are larger than the color tests indicate.

7. Losses

"Loss" of substance has been observed to occur even under the mild condition of paper chromatography. That is, the substance has been converted to another chemical species so that it does not appear in the expected location, or is not found by the test reagent upon which reliance has been placed. Some changes of this kind were discussed under double zone formation. Others may be caused by oxidation or other chemical reactions, or by heat- or light-induced changes, by acid- or alkali-catalyzed reactions.

The formation of pink "beards" on the ninhydrin-developed zones of amino acids run on paper containing traces of copper ion (191) are a manifestation of the formation of copper complexes of the amino acids. These run just ahead of the amino acid zones, and yield a pink color with ninhydrin. According to Dent (226), the sensitive amino acids cystine and cysteine are seldom seen in chromatograms at their proper places when present in the original mixture in small amounts. They are readily oxidized at the sulfur link.

Savard (811,812) has reported that steroids exposed to light over a long period during the drying of a chromatogram may undergo marked changes, during which steroid is lost. In a special experiment a zone of testosterone on paper was exposed to ultraviolet light ("Mineralite," 2 hr at 6 in.). The zone was eluted and chromatographed, whereupon at least six new presumed steroid areas appeared, with a corresponding decrease in the amount of testosterone.

The analyst must always be on guard against such occurrences as this. But the paper chromatographer needs to be especially wary because he uses dilute solutions and spreads small amounts of substances out over relatively extensive surfaces.

XVI. PRACTICAL CONSIDERATIONS

1. Advantages of Paper Chromatography

Paper chromatography is an extraordinary handy tool because the apparatus can be so simple, and because the actual time required in manipulations is usually small. Most of the time, the chromatography can proceed unsupervised. Closely related homologs can be separated, often quite sharply. A measure of the acuity of the method is the ability to separate steroids that differ only in conformation. Thus a steroid with an hydroxyl group equatorially oriented can be separated from its epimer in which that substituent is axially oriented (811).

The method is applicable to any mixture that can be brought into solution in a solvent that does not attack paper too rapidly, and in qualitative work, at least, the separations are not very sensitive to changes in the ambient temperature.

The results that can be obtained are on the whole highly reproducible, and this observation, plus the fact that one can work with minute amounts of material, makes it so that paper chromatography is coming to be the preferred method for examining all chemical mixtures: inorganic and organic, of industrial, biochemical, and medical, or whatever origin.

A zone of mixed amino acids might contain, say 0.1 to 25 μg. of each acid; of sugars, up to 30 to 40 μg. of each sugar; of steroids 3 to 30 μg.; of purines and pyrimidines 1 to 50 μg.; of phenols, 5 to 20 μg.; and so on (84). The method is obviously not preparative in the ordinary sense, though with improvement in techniques it is becoming possible either to handle, with improved assays, for example (156), such small amounts of materials, or to by-pass the need for "preparation" by doing all the necessary studies of the substances on the paper. The elegant analyses of peptides and polypeptides have shown the way, here.

2. Disadvantages of Paper Chromatography

Ordinary paper chromatography is not satisfactory as a purely preparative method.

XVII. LARGER SCALE WORK

The amount of material handled on paper can be increased—sometimes very much—by operating at an elevated temperature. For example,

Hough and co-workers (196,424), working at 60°C., with 1-butanol-water, were able to separate 0.3 g. of a mixture of D-galactose, D-xylose, and L-rhamnose in 18 hr., and larger amounts could be separated on cellulose.

By using thicker papers it is possible to handle more material than ordinarily. A thick paper like Whatman No. 3, for example, will hold considerable material. Cellulose sheets approximately $^1/_8$ in. thick were used by Cuendet, Montgomery, and Smith (207) for handling large quantities of sugars. The cellulose sheet, not being very strong when wet, was supported on glass and run in a horizontal position in a chamber made of a 100 mm. diameter glass tube closed at the ends with foil-covered stoppers. The paper, 5 cm. wide, rested on glass T-pieces inserted across the inside of the tube. The developer was led into the strip through a bridge of absorbent cotton. Fairly large amounts of sugar could be put on the sheets. For example, the authors say that D-xylose at a concentration of 20 mg./cm. moved as a discrete zone, and sugar mixtures could be separated. D-xylose, 100 mg., formed a zone 4.3 cm. wide; D-glucose and L-rhamnose, 14 mg. of each, were separated and formed zones 4.5 and 4 cm. wide, respectively. With some solvents the R_F values of these sugars were the same as on Whatman No. 1 paper; but with a butanol–ethanol–water solvent, they were higher.

Tatum and Adelberg wrapped many sheets of paper together to give increased capacity (914). Hagdahl and Danielson (213,359) report a 10×25 cm. column (available commercially) made from a tightly rolled strip of paper, that is capable of excellent and reproducible separations. For example, a mixture of 250 mg. each of glycine, alanine, and valine in 100 ml. 70% ethyl alcohol was separated by development with 3.8 l. of 70% alcohol in 48 hr. (Grycksbo column, see Appendix).

XVIII. PRECAUTIONS

Precautions that have already been discussed include loss of material and changes wrought during the development. To these should be added the losses due to penetration of materials into the cellulose fibers so that they cannot be easily recovered, and the very strong adsorptions that can occur from extremely dilute solutions.

Normally, losses are not great if due precautions are taken. *The method is not seriously limited by this factor.*

One precaution not often stressed is the toxicity of some of the substances used as developers (see above).

It hardly needs to be stressed that the smaller the amounts of material that are handled the more scrupulous must be the attention to the details of the method.

Recovery of material from paper chromatograms is usually quite good. Because of the minute amounts of materials that may be used special kinds of losses are to be anticipated.

XIX. CHROMATOPILE, CHROMATOPACK

By stacking filter papers together and clamping them, a column without walls has been produced (the "chromatopile" (646)). In an experiment reported by Drell (247) on the preparation of thymidine from sperm nucleic acid one fraction, representing sheets 250 to 375 of a 500-sheet pile yielded, after extraction and recrystallization, 0.9 g. of thymidine. Using a "chromatopack," consisting of a pack of 200 2 $\times$ 18 in. strips of paper, each containing from 0.75 to 1.75 mg. of mixture, aligned and clamped together and developed with the appropriate solution, Porter (745) was able to separate mixtures in amounts of 150 mg. of 3 dyes; 150 mg. of three tobacco alkaloids; and 350 mg. of three organic acids.

CHAPTER VIII

Adsorption Chromatography (Tswett-Column Analysis)

I. PRINCIPLE

The distribution process that brings about a separation occurs between a fluid mobile phase and a stationary adsorbed interfacial phase. The material in the adsorbed state is retarded. The more strongly adsorbed substances are more greatly retarded. (The only exception to this is the case in which the adsorbent is moved and the liquid remains "stationary"; see below). As Tiselius has pointed out, if the adsorbed phase were to increase sufficiently in thickness, the conditions for partition chromatography might be reached. If oriented multilayers were to be built up, the conditions for a crystallization would be approached.

A *good chromatogram* is judged with different criteria depending on the type of chromatography. Frontal analysis, a simple method without the development feature, is satisfactory if sharp fronts are observed and if they are clearly marked off from each other. In a good development analysis all the components of the mixture are completely separated in compact zones on the column with sufficient empty adsorbent between that they can be cleanly and separately collected, and with sharp fronts to the zones and little or no tailing. A good elution analysis has the same requirements, except projected into the eluent: the zones appear with sharp fronts and little or no tailing, discretely in a minimum volume of effluent with a sufficient volume of empty liquid between the zones that they can be cleanly collected. A good displacement is one in which the zones show sharp front and rear boundaries, with little tailing, or overlapping; and a good carrier displacement is one in which the units of carrier completely separate the zones, with the carrier material readily separable from the zone material by chemical or physical means.

From the practical point of view, emphasis must be placed also on rapid separation and ease of carrying out the various manipulations. The factors that influence these requirements placed on the various types of procedure—sharp fronts, decreased tailing, clean separation of zones, speed of analysis, and ease of manipulations—are discussed principally under Sections IV, V, and VI.

II. CLASSIFICATION

The earliest work with this method that was well defined, that is, in which the investigators knew what they were doing and consciously utilized the procedure of development, was probably done in the early years of the 20th century, notably by D. T. Day (1031), C. Engler (1006), and M. Tswett (947). Tswett's work was especially definitive. Exhaustive investigations have, however, brought to light many earlier studies that contained hints of, and similarities to, this work, and of course, as the definition of chromatography is imperceptibly changed by inclusion of additional methods under this name, the historical picture is affected. A great deal of study has been given to the history of chromatography, and of Tswett and his contemporaries, and of predecessors who had to do with aspects of the method (392,973,974,976,977,1006,1008,1030,1031).

Types of adsorption chromatography can be classified under headings depending on whether the mobile phase is a gas or a liquid; and whether the liquid mobile phase is applied to a column of adsorbent or to the center of a shallow bed, with radial development. By far the most common applications involve columns, with liquid mobile phases. In one case the solid adsorbent has been sifted into the solution, with separation of the mixture occurring, as the more strongly adsorbed material is collected in the lower layers of the sedimented adsorbent (569). This application is not further considered here.

The method may be utilized in a single, or essentially single, unit process, or a continuous process may be contrived (791a,897,898). Further, as pointed out in Chapter II, the mobile phase may be contacted with the stationary according to the procedures of frontal, development, elution, gradient elution, displacement, or carrier displacement analyses. Heat (954) or lowered temperature may be applied. The mobile phase may be forced through the bed of adsorbent by applied pressure, by centrifugal force, by gravity, or presumably by other means.

Gas adsorption analysis will not be discussed here since it appears likely that where gases or vapors are to be separated the gas partition method is the method of choice (Chapter V). The only exception may be in the separation of permanent gases. The subject has been reviewed by Patton, Lewis, and Kaye (717). The method has found industrial application under the name Hypersorption and Rotosorption (see Section XI).

This chapter will be devoted chiefly to adsorption chromatography using columns, and liquid mobile phases. Radial chromatography will be touched upon, and examples of all types will be given in Section VI.

III. APPARATUS

The apparatus used in column chromatography is closely like that already described for column partition, in Chapter VI, Sections III and VI. It can be very simple or more complicated, as shown there and in Section VI below. The complications are usually introduced in connection with collecting and analyzing the effluent from the column, and with controlling pressure at the head of the column, or changing the composition of the eluent in gradient elution analysis.

The chromatographic tube (Fig. VI-2) may be of any cross-sectional shape: circular is usual; square has been suggested for special purposes (60,459). The tube may be of glass; quartz (459); of a plastic such as Lucite (206); of cellophane or similar material (701); or of metal (206). It may be constructed so that it can be opened longitudinally (206) or laterally (60). It may be given a taper, with the slightly narrower end at the foot of the column, to aid extrusion (315). The adsorbent in the form of folded strips, disks of paper, or cloth may be piled up in a tube (539,1034), or clamped without the use of a tube (Chapter VII, Section XIX).

The size of tube chosen depends of course on the amount of adsorbent that will be used (Chapter XIV). (A full discussion of the factors that influence the choice of dimensions is deferred to the next section.) Examples may be derived from those in Section VI of this chapter. As a general rule, however, the column of adsorbent formed should be considerably longer than it is wide, say 10 to 100 times as long. The considerations regarding packing and pressure-drop, as well as separation of zones, are those in Chapter VI, Section VI. In general, very short columns, relative to their width, are useful only in the frontal analysis technique, though see below regarding sharpness of fronts (Section VI).

LeRosen (541) found that the velocity of flow of solvent through columns of calcium hydroxide was essentially independent of the diameter of the column. Diameters of 17, 43, and 70 mm. gave almost equal velocities of flow (mm./min.) for the same length of column.

Cassidy (159) found that columns prepared from 1 g. of charcoal each and packed in tubes 13, 16, and 23 mm. in diameter gave elution chromatograms with exactly the same concentration profiles when treated with 0.3 g. of lauric acid dissolved in 100 ml. petroleum ether and developed with petroleum ether. The rates of flow (ml./min.) were in the order 23 mm. tube $>>$ 16 mm. $>$ 13 mm. Weil-Malherbe (978) says that with a given quantity of adsorbent the ratio of length of column to diameter may be varied between about 5 and 20 without much effect on the concentration profile of the zone. Mair (593) found that the sharpness of

separation between aromatics and nonaromatics on certain silica gels did not depend significantly on the rates of flow, which were varied from x to $19x$. These factors were also investigated by LeRosen (See Chapter VI, Section VI).

The other accessories for chromatography, fraction collectors, etc., may be found listed, along with chromatography tubes, in the appropriate appendix.

IV. STATIONARY PHASE

1. Stationary phases of the greatest variety have been used in chromatography. Presumably any finely divided or porous solid which is not too soluble in the mobile phase may be used. Also, the chromatographic method may advantageously be used to determine the adsorptive behavior of a substance. Thus, for example, Swanson and co-workers (900) used columns of soils to study the adsorption of the insecticide Lindane.

2. The **particle size** of the adsorbent used in column chromatography exerts an important influence on the quality of the separations, as Tswett observed (947). He pointed out that large-grained adsorbents gave blurred chromatograms, and that fine powders gave best results. This has been noted repeatedly since, but it is difficult to give an absolute prescription about the "best" particle size.

Fairly wide differences are found in the particle sizes of the adsorbents used in chromatography. Zechmeister and Cholnoky (1035) list a number of adsorbents and their "average particle sizes." These vary from about 1.5 to 10.5 μ. Since 1 μ equals 0.001 mm., powders such as these would be finer than that which just passes a 325-mesh sieve, the openings of which are 0.043 to 0.044 mm. (518). Of one sample of a commercial alumina standardized for chromatographic adsorption analysis somewhat more than 30% passed a 300-mesh sieve. LeRosen (541) found a satisfactory range of V_c (V_c is defined as velocity of flow of developer solvent in millimeters per minute through the column when a state of constant flow has been reached) with calcium hydroxide columns to be associated with an average particle size of 5 to 15 μ. The range of V_c was 5 to 15 mm./min. Smith (865) recommends alumina passing a 325-mesh sieve. On the other hand, for laboratory use (see example, Section VI.3) Mair and Forziati find 28- to 200-mesh silica gel quite satisfactory, and for large-scale applications 24- to 42-mesh (595).

The question may be clarified by considering the factors involved in choice of appropriate particle size, or, more realistically, *range*. Recalling the requirements: sharp fronts, decreased tailing, clean separation of zones, speed of analysis, and ease of manipulations, listed in Section 1, it

becomes apparent that all are affected by the interrelated factors: particle size and size and shape of the chromatographic column. The clean separation of zones depends on the first two requirements, and the three will be classed together. The following have been observed or conjectured.

Apart from handling the apparatus, ease of manipulation depends largely on ease of packing the column. Manipulations in packing columns were described in detail in Chapter VII, Section VI. *In general, it is easier to pack columns homogeneously with larger diameter particles than with smaller.* A judicious range of sizes may be even better. Thus the commercial alumina designed for chromatography, referred to above, contained fines to the extent that 30% passed a 300-mesh sieve. The material was easily packed into apparently homogeneous columns by dry or slurry methods. Very coarse particles are undesirable (at least for laboratory use) because blurred fronts result; very fine particles and impalpable powders are undesirable because of their resistance to flow of developer and because of their tendency to channel and to pack unevenly. This leads to zones with irregular outlines which may in addition be slanted to the direction of flow of developer instead of being perpendicular to it, or may run faster at the outer portions of the column and slower toward the center, giving a cone-shaped appearance when the adsorbent is extruded and the zone sculptured out (1035).

A distinction between blurring and the frontal irregularities just described, and called "frontal deterioration," has been made by Hagdahl (358). The former is a lack of sharpness due to diffuseness *at the front;* the latter a condition due to irregularities in *the shape of the front.* These may be observed singly or together *in columns;* in the effluent they appear as a blurring only, since the effluent is mixed as it issues from the column, and a slanted front appears only as a slowly changing concentration in the effluent (see Fig. VIII-1). Large particle size adsorbents conduce to blurring; fine particle size adsorbents, because of difficulty in packing, to frontal deterioration.

Blurring, found with larger particles, may be due to accessibility factors. If the interstices between the particles are relatively large, then adsorptive in the center of an interstice will take a longer time to diffuse to the surface than would be the case with smaller particles and smaller interstices. Further, the developed surface in the interior of a porous adsorbent would be less accessible if the particles were large than if they were small. Both these accessibility effects imply that with larger particles flow rate may have to be slower for sharpest fronts to be obtained. Practically, however, optimum conditions for a separation require a balance between a rate of flow slow enough to give good fronts (still slower flow would usually give still better fronts) and fast enough to be conven-

ient yet bring about a separation. Obviously, substances with widely different adsorbabilities may be separated rapidly, with minimum concern for the shapes of the fronts (Example 1 in Section VI), whereas closely related substances require very slow flow, small particle size adsorbents

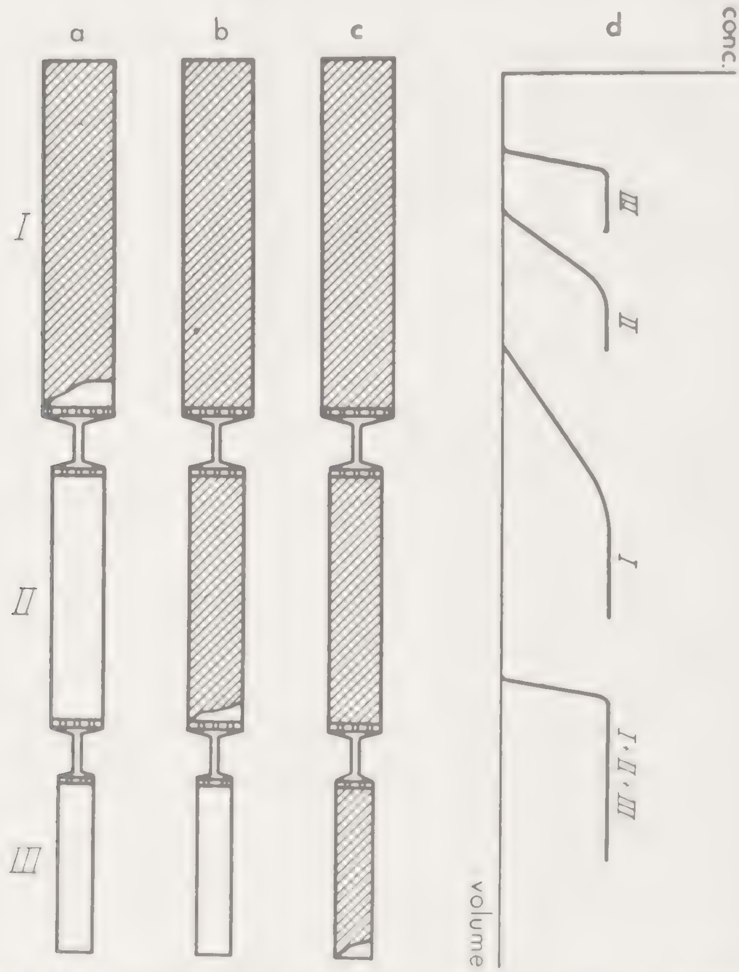


Fig. VIII-1. A three-component composite column at different stages of frontal analysis (*a*, *b*, *c*) and frontal analysis diagrams (*d*) obtained from the single columns I, II, and III used separately and in series. (By permission of the author and LKB-Produkter Fabriksaktiebolag, Stockholm). (From Hagdahl (358).)

and close attention to detail (Example from Moore and Stein. Fig. IX-4, Chapter IX, Section X).

3. In a study of the factors that affect fronts, Drake (243) found, with the charcoal Carboraffin Supra, adsorbing hydrochloric acid, acetic acid, citric acid, or ephedrine, that there was an initial rapid adsorption in which in less than 20 sec., two-thirds of the adsorption had occurred, and

that this was followed by a slower take-up which required from 1 to 20 or more minutes for completion. This suggested an initial adsorption to available surface, followed by a diffusion- and convection-controlled process of movement of adsorptive into the particles of adsorbent. (Drake suggests that this may explain why the steps in frontal analysis (break-through) curves often start sharply, while transition to the horizontal part where effluent concentration is unchanged is more gradual.) From his investigations, Drake concludes that the **velocity of flow** should be slow enough that the period taken for a front to emerge is relatively small compared to the total time of the run. Rates of desorption found were of the same order of magnitude as for adsorption, and support the conclusion **that velocity of flow in a column should be slow.**

But the slower the flow, the more opportunity for the disturbing effects of diffusion. Drake tested the effects of diffusion by using dyes (colored or fluorescent) on alumina or charcoal. He found that with an alumina column when development is stopped it may take an hour or more before the front of a zone shows a visual change in appearance, and a longer time with charcoal; thus it is possible, he concludes, that *a flow velocity "much lower than usual" may be tolerated before diffusion-caused diffuseness of the front becomes important.* Further, the lateral spreading of a developed irregularity, postulated by Claesson (177) as aiding the sharpening of a front, is made unlikely as a general thing.

The velocity of flow may, however, slow down during a run, or vary somewhat with the passage of zones out of a column. This has been a common observation. One cause of slowing down may be *swelling of the adsorbent particles*, an effect observed often in exaggerated form with ion exchangers, and correctable by preliminary equilibration of the particles with the mobile phase or by backwashing of the column. Glass tubes have been quite violently shattered under the force of such swelling. Another cause is *compacting of the column* when it is operated under pressure. This can to some extent be avoided by mixing the adsorbent with a filter aid such as kieselguhr (884) or paper pulp. The latter is especially effective with charcoal (963,964). Still another cause may be the *formation of a concentrated zone*. For example, as Drake pointed out, a gram of active charcoal may adsorb 0.3 g. of phenylalanine from an 0.5% aqueous solution (931). The increase of stationary material in the zone would be expected to exert some blocking effect. Cassidy (161) found that a gram of Darco G-60 charcoal will retain about 0.2 g. lauric acid from 0.0075 *M* solution in petroleum ether, and 0.4 g. stearic acid from a 0.0054 *M* solution in petroleum ether. These causes of slowing down may sometimes be countered mechanically by some device that controls the pressure on a column so as to ensure a constant speed of **flow (243).**

4. **Constancy of flow** is important. Thus in the case of frontal analysis of a peptone fraction on charcoal when the velocity of flow was decreased for a period and then returned to the original (by changing the pressure from 3 kg. to 1 kg. for a while and then back to 3 kg.), a break was observed in the effluent concentration curve (243). During this period the concentration of the effluent decreased, evidently because equilibrium had not been reached in the front, and the slower flow gave more time for adsorptive to become adsorbed. This effect was also observed with amino acids and monosaccharides. It was therefore concluded that *it is often deleterious to interrupt an experiment and continue it later.*

5. Another factor of importance to front sharpness is **uniformity of packing.** Drake has found that the irregularities or unevenness across the column of moving fronts are normally large compared to the diffuseness or lack of sharpness of the front itself. He defines a measure of unevenness of a diffuse front, whether horizontal or oblique, in terms of "percent distortion." In the column this is defined as the length of the front along the column, in millimeters, times 100, divided by average distance in millimeters that the front has moved. In the effluent the measure is front length in milliliters $\times$ 100 divided by retention volume in milliliters (243).

In many runs, using standardized alumina, or charcoal, the best results obtained (with charcoal) were a percent distortion of 5, and again an observation was made that was emphasized by Zechmeister and Cholnoky (1035), namely, that the shape of a zone *in* a column cannot be judged with certainty from the shape of the band of color around the outside of a column.

In this connection, Drake also studied the effects of *columns of different shapes.* He tested funnel-shaped columns, 20 mm. diameter at the top, 50 and 30 mm. high, and 2 mm. at the bottom, with stems 20 and 30 mm. long by 2 mm. internal diameter; a column 60 mm. high, 8 mm. diameter, with 240 turns of a 0.1 mm. deep thread in the wall; a conical column. The sharpness and evenness of the fronts were not improved by the use of such columns and they were difficult to make and pack. *Cylindrical tubes therefore seemed most suitable for columns.*

6. An interesting observation was made that the factor that determines the appearance of a front is chiefly the quotient distance of movement of front $\div$ diameter of column (see Fig. VIII-2). This indicated, as has been generally observed (243,1035), that the narrower the column for a given mass of adsorbent, the better the definition of the zones in terms of sharpness of fronts. *With alumina, Drake found a ratio of column length to diameter of 100:1 to be most suitable, and with charcoal, 30:1.* He found no essential difference between wet and dry packing, except that of convenience. Wet packing gave columns that were easily made free of gas bubbles; dry packing often gave trouble in this respect.

Besides the effect on flow rate of *sudden change in pressure*, referred to above, there may be caused a shift in the adsorbent. This can make fronts and zones irregular, as well as affect flow rate. Also, sudden changes in pressure were observed by Drake (243) to change the thickness of the film of liquid between the outer surface of a charcoal column and the inner wall of the chromatography tube, thus affecting the regularity and diffuseness of the zone.

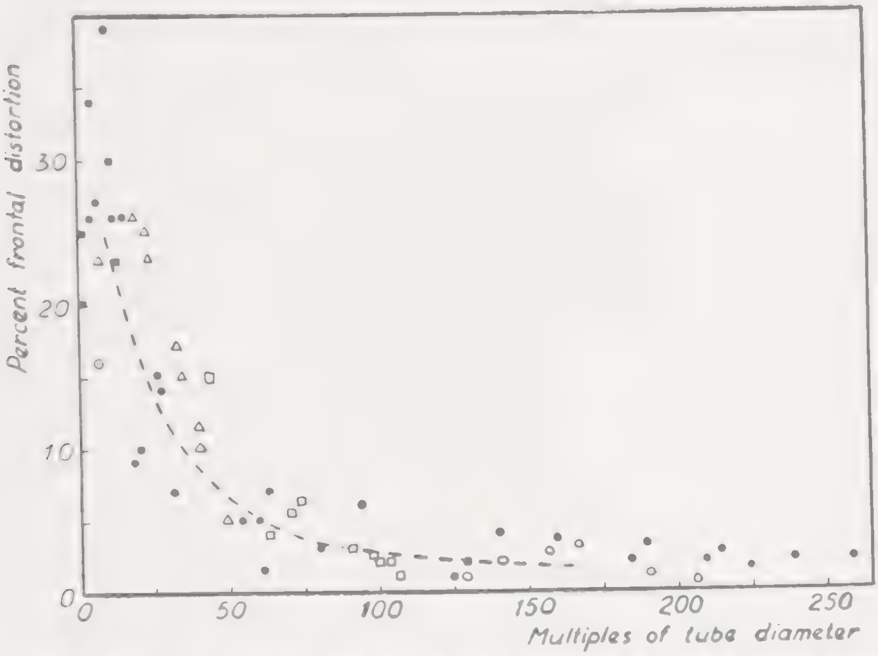


Fig. VIII-2. "Percent frontal distortion" as a function of the distance, measured in multiples of the actual tube diameter, that the fronts have moved. The tube diameters are ○, 1 mm.; ●, 2 mm.; □, 8 mm.; △, 10 mm., ■, 16 mm. (From Drake (243).)

We see then, in summary, that having decided on the kind and amount of adsorbent one wishes to use (Chapter XIV), in general the following considerations will lead to optimum separations. The particle size of the adsorbent should not be too large; if it is for any reason very small, an inert filter aid should be mixed in. The column should be formed in a tube considerably narrower than it is long. If a great deal of adsorbent is required, necessitating a large-diameter column, then one or more smaller diameter front-sharpening columns of the same adsorbent, connected by small-bore coupling tubes, should be attached at the exit of the large one. The optimum rate of flow may have to be determined, but in general the time required for a front to emerge should be small compared with the duration of the run. The experiment should not be interrupted, nor should the velocity of flow of developer be changed suddenly during the run.

V. ADSORBENTS

A set of tables, with attendant discussion of types of adsorbents, is given below. The volume of literature is so large that entire books have been written on single types of adsorbents. These are referred to where possible. The listing is probably incomplete, extensive though it is.

1. Aluminas and Related Materials

Aluminas suitable for use as adsorbents (see Table VIII-1) are usually made by partial dehydration of the hydroxide, which may be prepared by

TABLE VIII-1

Aluminas and Related Materials Suitable as Adsorbents

Aluminum Company of America
F-20, through 80, on 200 mesh
XF-21 $\leqslant$2.5% + 100, 5–20%; −325 mesh
H-51 microspheroidal $\leqslant$3.5 + 100; 25–35%; −325 mesh
Alupharm Chemicals
Basic, pH 10
Nonalkaline, pH 7.5
Acid, pH 4
All are activity grade I (Brockmann) and may be deactivated by addition of water.
Bio-Rad Laboratories
Basic, AG 10, 50–100, 100–200, minus 200 mesh
Neutral, AG 7, same mesh ranges
Acid, AG 4, same mesh ranges
Minerals & Chemicals Corp. of America
Activated bauxite (380)
Most laboratory chemical supply houses supply "alumina" or aluminum oxide.

hydrolysis or neutralization of the salts. "Fibrous alumina" (691,1019) is prepared from the cleaned metal by amalgamating it, and reacting with water, water vapor, or steam. Alumina, which is active as an adsorbent, always contains some water. Moreover it should not be reactivated at too high a temperature, because if all the water is driven off structural changes occur ("sintering") and the alumina loses activity. The surface area of alumina is said to remain unchanged in extent up to 528°C; and to drop by 40% after heating at 938°C. The nature of the surface may undergo changes during heating (796). Brockmann and Schodder (118) set up an arbitrary gradation of activities of alumina based on the chromatographic separation of certain dye-stuffs; a highly active commercial alumina was deactivated to certain degrees by exposing it to moisture for given times. (See Section VI of this chapter.)

A typical analysis of "Activated Alumina," grade A, is Al_2O_3 92%, loss on ignition 7%, Na_2O < 1%, SiO_2 < 0.1%, Fe_2O_3 < 0.1%, TiO_2 < 0.01%. The sodium is combined with the alumina and silica, and is not leachable. ("Activated Alumina" is a trade mark of the Aluminum Ore Co.) Some aluminas are said to contain sodium bicarbonate and carbonate (853).

Alumina has enjoyed the position for some time as the first choice for chromatography of new mixtures. It has probably been more used than any other adsorbent. It is a good adsorbent for most substances (with the proper solvents), has a large capacity, is white, insoluble, reasonably chemically inert (though the surface of the ordinary alumina gives a basic reaction), and readily obtainable. Alumina is, in fact, a very versatile adsorbent.

Alumina can be made to exchange anions or cations with suitable preliminary treatment. Kuhn and Wieland (508) prepared an anion adsorbent by treating commercial alumina as a slurry in water, with 2*N* hydrochloric acid with thorough mixing, to a distinct Congo red reaction. The alumina was then separated and digested with distilled water until the wash liquid just gave a weak violet color with sensitive Congo red paper. On this adsorbent these investigators were able to separate (chromatographically) a physiologically active pantothenic anion. This substance could not be taken up on sulfuric acid-treated adsorbent because the sulfate ion was more strongly adsorbed to the alumina. It could be eluted with alkali, which led these workers to set up the adsorption series for the acid-treated alumina: $Cl^- <$ active anion $< SO_4^{--} < OH^-$. Alumina may also be used as a cation exchanger, as has been shown by Schwab and Jockers (340,836,837). Some of the physical and other properties of alumina and related substances are discussed by Heinemann, Krieger, and McCarter (380) and by Krczil, and in other places (496,604), such as in the technical literature of the Aluminum Ore Co., and Bio-Rad Laboratories, and M. Woelm (see Appendix). The exchange capacity of alumina is low: *ca.* 0.01 meq./g.

Dr. B. F. Stimmel (881,882) reports that he can tamp 18.5 g. of dry 100- to 200-mesh alumina to a height of 21 cm. in a 13-mm. O.D. pyrex tube. The same batch of alumina can be tamped to 24 cm. by slurrying with dry benzene. Upon drying at the end of the experiment the alumina can be tamped to 22 cm. height. A column of 200-mesh alumina, mixed 4:1 with Celite No. 545, measuring 24 × 4.2 cm., was used to chromatograph 1 g. anthrone (735). Columns of 5 and 25 g. alumina packed by the slurry method in tubes 0.8 and 1.7 cm. diameter, respectively, gave columns 12 cm. high, and were used to chromatograph polysaccharide, taking a weight ratio of polysaccharide to adsorbent of 4×10^{-4} to 20×10^{-4} (290).

2. Charcoals and "Carbons"

The term carbon (Table VIII-2) is widely used in industry to refer to activated charcoals which may contain considerable ash. The use of this

TABLE VIII-2
"Carbons," Charcoals, and Carbonaceous Adsorbents

Acheson Colloids Co.
- A large variety of dispersions of colloidal graphite, dispersed in water, oil, glycols, etc.

American Norit Co., Inc.
- Norit RBI and RHSI are said to be most suitable for gas adsorption.
- Norit ROK, or PC III is used in chromatography of liquids. For decolorization, Norit A, B, F, and SGII Extra.

Atlas Powder Co.
- Darco G-60, from pine charcoal, highly purified by acid and water washing, ash 5–8%, water-solubles 0.1–0.2%, pH 5.5–6.5. 100% through 200 mesh; 70% through 325 mesh
- Darco S-51, from lignite, purified, ash 18–23%, water-solubles <1.0%, pH 5–6. 100% through 200 mesh; 70% through 325 mesh

Baugh & Sons Co.
- Bone char, low area, large-pore adsorbent, pore volume *ca.* 0.3 c.c./g.
- Synthad

Bell Telephone Laboratories
- Polymer Carbon, from dehydrogenation of polydivinyl benzene spheres

Cliffs Dow Chemical Co.
- Cliffchar (from wood charcoal), typical, ash, 4.0%, water-soluble 1.8%, pH 10.4, powder, 100% through 325 mesh

Chemical Process Co.
- Duolite S-30 and S-35, resinous adsorbents

National Carbon Co.
- "Columbia" activated carbon, Grade L, 20–48 mesh

The Permutit Co.
- Permutit Carbo-Dur (from bituminous coal), screen size 20/50 (coarse sand)
- Permutit DR, a decolorizing resin

Pittsburgh Coke and Chemical Co.
- Pittsburgh Activated Carbons type BPL for vapor phase applications; and types SGL, and CAL for applications to liquids, may be obtained in various mesh sizes.

West Virginia Pulp & Paper Co.
- Nuchar (from pinewood charcoal)
- Suchar (from paper-pulp waste liquors)

Blood charcoals, sponge charcoals, various kinds of wood charcoals, sugar charcoal and some of the above charcoals can be obtained from Laboratory Supply Houses.

term is apparently based on the observation that when the carbon is burned off a decolorizing "carbon" such as bone charcoal, which may contain 90% ash, the ash residue shows little or no decolorizing power. The charcoals and carbons run the gamut in composition from nearly

pure carbon purified graphites and sugar charcoals) to charcoals containing approximately 90% ash (bone chars). Ogawa (693) determined the elementary composition of a sugar charcoal to be C, 95.2 to 95.3; H, 0.7; O, 4.0 to 4.1%. Mantell (604) has given an analysis of American bone char (before use) as carbon 9.30, sand, etc., 0.42, tricalcium phosphate 75.00, calcium carbonate 6.23, calcium sulfate 0.08, calcium sulfide 0.01, ferric oxide 0.23. Bone char usually contains combined nitrogen and some moisture. Studies have indicated that the decolorizing power of bone char may remain at a maximum at carbon contents varying between 2% and 9%, but the situation is extremely complex and cannot be adequately treated here (32–34). Charcoals from animal and vegetable sources retain some of the cellular structure of the original material unless they have been graphitized by excessive heating.

Even charcoals made from recrystallized sucrose or dextrose contain ash. This ash may be removed, at least to some extent, by the method of Miller (639). The charcoal, ground to pass a 300-mesh sieve, is mixed in a platinum vessel with concentrated hydrofluoric acid to form a thin paste. This is warmed gently until most of the acid has been driven off and is then heated until the hydrofluoric acid is completely removed. The adsorbent is then boiled with concentrated hydrochloric acid, the suspension diluted, and filtered in a Büchner funnel through hardened filter paper. The treatment with hydrochloric acid is repeated. The charcoal is then washed by repeated digestion with distilled water, dried, and ignited in the absence of air. The whole process is repeated until the desired purity is attained. The fine grinding is an essential. Miller gave the following examples of reduction of ash content: with blood charcoal the original ash content of 8.39 was reduced by three treatments to 0.06%; with Norit, the original 6.47% was reduced to 0.04% in two treatments; with activated sugar charcoal the initial ash content of 0.1% was reduced to 0.00% by one treatment. Miller (640) made an extensive study of purified charcoals from different sources and found them to become all much alike in some ways with purification.

A great deal has been written on the nature of the charcoal surface and the methods of modifying it. The surface can be modified by suitable treatments so that it is organophilic or hydrophilic (488). Bartell and Lloyd (37) have prepared a charcoal, by activating purified sugar charcoal in a highly oxidizing atmosphere below 150°C., which approached the behavior of hydrophilic silica in its adsorptive properties; it gave only negatively charged particles when suspended in water. Their "high-temperature charcoal" activated near 1000°C., behaved as an organophilic adsorbent and on suspension in water showed both positively and negative charged particles. The surface of charcoal may also be modi-

fied by coating it with a film of fatty acid or other substance, as is discussed in detail in Section VI, below. The extensive work which has been done with carbonaceous adsorbents cannot be discussed here (187,220, 220a, and especially 250a,475a).

Charcoal that is used for adsorbing nitrogen-containing compounds such as aromatic amino acids must be freed from soluble nitrogen material. This was done by Schramm and Braunitzer (822) by bringing the adsorbent to a boil in a 5- to 10-fold amount of 20% acetic acid, filtering hot, and washing with hot water until no nitrogen was found in the wash liquid. This usually required two or three washings. The charcoal should also be treated, as Tiselius showed (929), to suppress its deaminating property. This can be done (822,825) by suspending the charcoal in water and treating it at *ca.* 60°C. for several minutes with 50 mg. KCN for each 100 g. charcoal. The product is then thoroughly washed. Blackburn and Kipling have discussed the effects of ash in charcoals (79).

An interesting substance called "Polymer Carbon" has been prepared by Winslow and co-workers at the Bell Telephone laboratories (1012, 1013). They copolymerize divinyl benzene with ethyl vinyl benzene and carbonize the resulting beads to produce high surface area adsorbents.

Holman (415) has suggested an easy way to clean hands soiled by charcoal. The hands are first moistened thoroughly with an oil, then washed with soap and water. The oil functions to displace the charcoal from the skin, which soap and water may not succeed in doing.

3. Clays and Related Substances

The clay minerals (Table VIII-3) comprise a wide variety of substances some of which are, or may be converted into, adsorbents. The minerals are crystalline substances of colloidal dimensions which show exchange properties to varying degrees. The behaviors of clays are related to the size, shape, and composition of the particles present, and to their exchange properties (376). The size of the particles being within colloidal dimensions, the substances show relatively large surface areas. A mica from soil may have a surface area of some 60 m.^{2}/g., and a kaolinite 80 m.^{2}/g. (384) (see Table VIII-14). Some natural clays will have particles larger than others, and the size can always be changed by physical treatment in activation and other processes. The shapes of the crystalline particles can be shown by electron microphotographs (1,605,845). Kaolinites and some bentonites are composed of platelets. Montmorillonite is composed of platelets. Attapulgite is made up of fibrous particles. In composition, these substances are silicates. The kaolinites are aluminosilicates. The bentonites are also aluminosilicates, but more complex. They are of

TABLE VIII-3
Clays, Earths, Bentonites, etc.

The American Colloid Company
- Volclay bentonites, very small particles; strongly hydrophilic

Filtrol Corporation
- Filtrol clays, particularly used with organic materials, 100% through 100 mesh, 73% through 325 mesh

Floridin Company
- Florida fuller's earth (floridin), base exchange capacity 25–30 meq./100 g. pH 7.9–8.2 in distilled water; surface pH *ca.* 1.5
- "Florex," various mesh sizes available "B" 16–30 mesh; "S" 30–60; "XXS" 60–100; "XXF" 90% through 100; "XXX" 90% through 200 mesh
- "Florigel," 60% through 200 mesh
- "Diluex," various mesh sizes available
- Activated bauxite
- "Florisil," synthetic magnesia-silica gel; pH of slurry 8.5, various mesh sizes available, from 30–60 to through 300

Columbia-Southern Chemical Corporation
- Silene EF

Industrial Minerals & Chemical Company
- Bleaching clay #260, 95% through 200 mesh, pH value 4.0

Minerals & Chemicals Corporation of America
- Attapulgus fuller's earth products
- Attasorb, finely divided material
- Attaclay, Permagel, Attasol
- All have pH value 7.0–8.0

Fuller's earths and talcs can be purchased from most laboratory supply houses.

Tamms Industries, Inc.
- No. 200 fuller's earth, 98% through 100 mesh; 65% through 325 mesh; pH value 5.0, 1/2% moisture; a hydrous aluminum silicate

different types: some have sodium, others calcium as the chief exchangeable cation, but potassium and magnesium are also present. The behavior of these substances toward water, electrolytes, and organic liquids depends very much on their composition (376).

The crystalline base-exchanging silicates have been classified by Hendricks (384) in terms of the site of the exchangeable ions. The original article should be consulted for the complete classification, which is not given here. Among those crystalline silicates with exchange sites within the structural framework are the zeolites. These have an open framework of interconnected channels, so that when an ion diffuses into the structure another can diffuse out, thus maintaining electroneutrality. The dimensions of these channels are different for different zeolites. Barrer has used this property to divide zeolites into three classes which differ in the size of molecule that can be occluded by the particular zeolite. The cations required to balance the charges of these substances

are located, along with their associated water molecules, in the interstitial spaces of the framework. The other class of crystalline silicate exchangers have the exchange site external to the structural framework. These fall into three subclasses: (*a*) the montmorillonite type of clay minerals wherein the exchange sites are mostly on an inner surface accessible to swelling; (*b*) the micas (including glauconite), in which the exchange sites are at the limiting crystal surfaces; and (*c*) the kaolin minerals wherein only a limited part of the external surface supports exchange. In attapulgite, which falls between several classes, the fibrous structure leaves channels of cross section about 3.7 by 6.0 A (605). Molecules of considerable size can be admitted. In the natural clay these are largely filled with loosely held water, and a relatively small proportion of mobile (exchangeable) cations. An attapulgite might show a base exchange capacity of 0.25 to 0.3 meq./g. (376). In the kaolinites the base exchange occurs, so to speak, where the ionic lattice terminates or where the platelets have been broken, so that an incomplete balancing of charge requires the presence of external ions (384). The exchange capacity compared with the other minerals is somewhat low, for this reason. Thus the kaolinite referred to above, with a surface area of some 80 $m.^2/g.$ shows an exchange capacity of about 0.12 meq./g. An attapulgus clay might show a surface area of 170 $m.^2/g.$ (see Table VIII-14).

Fuller's earth and other clays are used in extremely large quantities for bleaching, decolorizing, catalysis, and a myriad of other applications. (Note that British fuller's earth contains 20% of calcite, and is bentonitic; U. S. fuller's earth is attapulgus clay. Bentonite is the ore of montmorillonite mineral, and the name is given if this is the major constituent.) In using these substances in the laboratory for analytical work it may be necessary to wash off traces of oily material which may be present (1038). Sometimes a prewash (see below) with an organic solvent can improve the behavior of the adsorbent. Thus, Binkley and Wolfrom (75,76) prewash Florex xxx with an azeotropic mixture of dioxane and water.

A great deal of very interesting chromatography has been done with clays as adsorbents. For example, remarkable separations among sugars and related substances have been carried out by Wolfrom, Binkley, and their co-workers. These have been reviewed by Binkley (75), who has included an extensive table showing the adsorption series on the fuller's earth clay Florex xxx. The use of clay adsorbents for development chromatography suffers because the columns (usually made up with 15% to 20% by weight of kieselguhr to aid flow rate) are difficult to extrude. However, this is overcome to some extent by the use of a slightly tapered tube, wider at the top (76). Sometimes, too, with aqueous developers,

fine clay particles are peptized. Inorganic materials may be leached from the column. The suspensions so formed are clarified by treatment with charcoal, vacuum filtration through acid-washed asbestos, and gravity filtration through hardened paper (1020). Despite these drawbacks the method, as exemplified by these workers, is a powerful one.

Talc has shown itself to be a good adsorbent for separating a number of sulfonated azo dyestuffs, using aqueous pyridine as the developer (366). Bentonite has shown itself to be the best adsorbent (better than Florisil, magnesia, Nuchar C, alumina, or silica gel) for separating phthalans from aromatic and saturated hydrocarbons obtained in investigations of coal degradation products (271).

4. Kieselguhrs. Diatomaceous Earths. Filter Aids

Ever since their introduction by Strain (884) in this application the diatomaceous filter aids (Table VIII-4) have seen increasing use in

TABLE VIII-4
Kieselguhrs, Diatomaceous Earths, Filter Aids

Great Lakes Carbon Corporation, Dicalite Division	
	Dicalite, diatomaceous earth, largely silica in composition; grades, Dicalite 115, Speedflow, Speedplus, and 4200
Johns-Manville Corporation	
	Celite filter aids; diatomaceous earth, largely silica in composition; grades, Filter-Cel; Hyflo Super-Cel; Super-Cel; Celite 503; Celite 545.
Tamms Industries, Inc.	
	Multicel diatomaceous earth of extreme fineness, not recommended as a filter aid; grades 600, 98½% through 325 mesh; 617, 95–98% through 325 mesh; 680, 98½% through 300 mesh.

chromatography. They have been used in three applications. One is for improving the solvent flow rate through columns of adsorbent. The filter aid is mixed with the finely powdered adsorbent in a suitable ratio. LeRosen (542) found an inverse linear relation between the volume fraction of silicic acid in mixtures with Celite 535 and the $\log_{10}$ of the rate of flow of developing solvent. The use of a filter aid mixed in with the adsorbent also makes it easier to pack the adsorption columns uniformly. Using a standard method of packing in which the adsorbent was poured slowly into the tube under the full vacuum of a water pump, and the tube then tapped vigorously, LeRosen (542) found that the percentage of the tube volume occupied by the adsorbent increased linearly with the weight fraction of silicic acid in admixtures with Celite 535. The ratio of filter aid to adsorbent for a given new application is usually determined by trial. Wilkes (997) has recommended Celite 545 as a high-speed filter aid.

The adsorptive power of filter aids for most substances is relatively low, and usually does not interfere with their use as filter aids. In new applications, however, possible adsorption by the filter aid should be taken into account (892,997). Also, impurities that may wash off of filter aid should be looked for (885,922). It is best to use analytical-grade filter aids in chromatography. Filter aids are finding considerable use as supports for the stationary phase in gas partition chromatography.

Filter aids may be used as adsorbents in certain cases. Gallagher, Koch, and Dorfman (309) adsorbed the male sex hormone from acidified urine with Dicalite. Estrogenic substances were not appreciably adsorbed. Since this early work, kieselguhrs have been found to be very useful adsorbents for high molecular weight substances such as proteins (182, 216,580,588,624,1041) and subcellular granules (780–782). Clauser and Li (182) have found, using a purified, acid-washed kieselguhr, Hyflo Super-Cel, that many proteins including bovine serum albumin (BSA), methylated BSA, and the growth hormone somatotropin could be adsorbed at a pH below the isoelectric point, and recovered with yields of 80% to 90% and no evidence of extensive denaturation by eluting at a pH above the isoelectric point. Mixtures of these proteins could be separated. It seems evident that for applications of this kind, where sensitive substances are being separated, an inert, weak adsorbent like kieselguhr has interesting possibilities. Tiselius (934) has suggested that in work with easily denatured substances strongly hydrophilic adsorbents seem to show less tendency toward surface denaturation than more hydrophobic substances.

5. Silicas and Silica Gels

These substances (Table VIII-5) are of considerable use in chromatography. Silica gel has been used extensively in the separation of paraffin, naphthene, and aromatic hydrocarbon fractions by Mair and his co-workers (593,595,596). A typical analysis of a gel prepared for chromatographic use by the Davison Chemical Co. is, on the dry basis, 99.71% SiO_2, with traces of iron, aluminum, titanium, calcium, sodium, zirconium, and other elements present. The screen analysis of the gel is given as on 100 mesh $< 5\%$; on 200 $< 20\%$; on 325 $> 75\%$. The total volatile material: 6.0%. Santocel is a silica aerogel (480) made in several grades. The approximate analysis for most grades is: SiO_2, 81.5 to 90%; volatile (water, alcohol, and acetaldehyde) 7 to 10%; Na_2SO_4, 8.5 to 9.5%; Al_2O_3 and Fe_2O_3, 1%. The substance is light in weight and has a large surface area (Table VIII-14).

A study by Fink and co-workers (284) of silica gel and other adsorbents used for separating the components of cracked gasolines yielded the

TABLE VIII-5
Silica Gels and Silicic Acid

Davison Chemical Company

Grade 912. 28–200 mesh. Pore volume 0.45 cc./g. Average pore diam. 22 A.

Grade 922. Through 200 mesh. Pore volume 0.45 cc./g. Average pore diam. 22 A.

Grade 923. 100–200 mesh. Pore volume. 0.4 cc./g. Average pore diam., 32 A.

Grade 950. 60–200 mesh. Pore vol. 0.4 cc./g. Average pore diam., 32 A.

Grade 963. Through 325 mesh. Pore vol. 0.45 cc./g. Average pore diam. 22 A.

Grades 912, 922, 963 generally are used for hydrocarbon and steroid separations.

"Grades 923 and 950 are extremely pure silica gel that minimize olefin polymerization, sometimes experienced with regular grades of silica gel, in hydrocarbon separations.

They are also used in steroid separations." All this information is quoted, with permission, from the industrial literature.

Monsanto Chemical Company

Santocel, a light, fluffy silica aerogel. Average particle size of Santocel 54 is 0.5–3.0 μ.

Silicic acid and silica gel for chromatography can be obtained from most laboratory supply houses.

following data. The capacity of various adsorbents was determined at about 25°C. by adsorption of toluene from 20% by volume solution in isooctane, and stated in milliliters toluene adsorbed by 100 g. adsorbent. Davison gels showed capacities of 14 to 20.3 ml.; an alumina, 4 ml., a bauxite, 5.5 ml., a bentonite 2.9 ml., a fuller's earth 4.5 ml. The capacity of the silica gel was not appreciably related to the particle size, in tests with 28 to 200 mesh, 60 to 200, 100 to 200, and through 200. Essentially the same volume capacity of about 20 ml./100 g. of gel was shown for toluene, xylene, *n*-propyl benzene, cumene, *tert*-butyl benzene, *p*-cymene, and phenylcyclohexane. The capacity of the gel for olefins of about the same molecular weight increased in the order straight chain < branched chain < cyclic, the average capacity being about half that for toluene. The gels were "tempered" at about 100° to 110°C. to decrease polymerizing effect. It was found that the volume of solution that could be separated by a given column increased by about 0.4% per degree lowering of temperature of the run, and running at lowered temperature also aided in suppressing polymerization of olefins.

Silica, or silicic acid, reagent grade, is finding a great many applications in chromatography. The substance is inert and a good adsorbent for polar substances. It was found in Zechmeister's laboratory (826) that the strength of the adsorbent can be influenced by prewashing. The prewashing, which is most effective, appears to remove water from the adsorbent (542). The order of increasing adsorptive power produced by prewashing has been given by Schroeder (826) as follows, where V ml. is

the volume of solvent which is required to wet completely the column of adsorbent; alcohol-prewashed adsorbent (V ml. absolute alcohol followed by $2V$ ml. ligroin) less active than unprewashed adsorbent, which is less active than ether-prewashed adsorbent (V ml. ether, $2V$ ml. ligroin), which is less active than acetone-ether–prewashed adsorbent ($0.2V$ ml ether, V ml. 1:1 acetone–ether, $0.8V$ ml. ether, V ml. ligroin). The acetone–ether prewash yields an adsorbent of the same activity as is produced by heating the silicic acid at 200°C. for about 40 min. LeRosen (542) found that Merck reagent silicic acid was very responsive to this activation by prewashing technique.

6. Additional Inorganic Substances Used as Adsorbents

The substances listed under this heading (Table VIII-6), with a few exceptions, are not so frequently used as those described previously.

TABLE VIII-6
Additional Inorganic Substances Used as Adsorbents

Columbia Chemical Division, Pittsburgh Plate Glass Company	
	Silene EF, a synthetic, hydrated calcium silicate
Food Machinery & Chemical Corp., Westvaco Chemical Division	
	Micron brand of magnesium oxide, No. 2641
	Magnesol, a synthetic hydrous magnesium trisilicate
Linde Air Products Company	
	Molecular sieves; Inorganic substances capable of taking up small molecules but not large ones.
U. S. Gypsum Company	
	Alba-floc. A finely divided calcium sulfate.
Many other substances that may be used, such as barium carbonate, calcium carbonate (U.S.P. pptd. chalk), and calcium hydroxide, can be obtained from laboratory chemical supply houses.	

The exceptions are Magnesol and Silene EF, which have found much use in the separation of sugars and their derivatives (75,76), and Micron brand of magnesium oxide, which Strain found very useful with plant pigments (886,888).

A number of water-soluble inorganic substances can be used as adsorbents with good effect, as Tswett showed. Brockmann (113) has separated azobenzene dyestuffs on anhydrous copper, zinc, manganese, aluminum, and magnesium sulfates, sometimes obtaining separations that could not be obtained on alumina. Hydroxyanthraquinones, also, could be separated. The organic materials were easily recovered by dissolving the adsorbent in water and extracting with an organic solvent.

7. Organic Substances Used as Adsorbents

A number of organic substances have found use as adsorbents (Table VIII-7), particularly in applications where mildness of action is desired. Filter paper pulp may be purchased, or it may be prepared by boiling and shredding filter paper scraps (with a hand or mechanical beater). Filter paper pulp makes an effective filter aid for charcoal adsorption columns (964). It can be used as a support for one phase in the partition chromatogram (Chapter VI). Boscott (95) has suggested that cellulose acetate (1/4 in. staple filaments) can be used in this way for supporting the butanol phase in partition chromatography. The polymer is swelled

TABLE VIII-7
Organic Substances Used as Adsorbents

Amend Drug and Chemical Company	
	White potato starch
Brown Company	
	Solka-Floc BN 200, a purified wood cellulose
Chemical Process Co.	
	Duolite S30, S35; resinous adsorbents
Grocery Stores	
	Confectioner's sugar, multiple X; Contains about 3% starch
Laboratory Supply Houses	
	Filter paper powder and tablets (see Table VII-4)
	8-Hydroxyquinoline
The Permutit Company	
	Decolorizing resin, mesh size 90% −20 +50. Reddish brown granules, used for decolorizing a wide variety of materials.
Tennessee Eastman	
	Oxycellulose; oxidized cellulose

with butanol at 50°C. for 30 min. and then the excess solvent is removed by filtration, pressing, and washing with saline. Starch has also been used in partition chromatography (see Chapter VI). Starch also can act as an adsorbent (535,648). Confectioner's xxxxx powdered sugar is a satisfactory adsorbent for many separations (888,1035).

Cellulose columns have been used to separate cellulytic components in microbial cellulases (317). Oxidized cellulose has been used to investigate pituitary extracts (21).

8-Hydroxyquinoline (272,273,783,784) may be mixed with an equal weight of starch and ground to pass a 60-mesh and remain on an 80-mesh screen (meshes to the linear inch). This is packed in the chromatographic tube by tapping the tube after small additions of adsorbent, not by tamping. This was used to separate an inorganic mixture. Control of pH was found to be important.

8. Impregnated Papers

Alumina-impregnated papers have been used in the separation of inorganic ions (293a,294), vitamin A, and carotenoids (215), and steroids (85,149,215). The paper can be prepared (852) by briefly immersing sheets of Whatman No. 54 filter paper in aluminum sulfate solution (13 g. $Al_2(SO_4)_3 \cdot 18H_2O$ in 100 ml. water), draining, and exposing to ammonia vapor for 15 hr. After this period the papers are washed continuously for 6 hr. (tap water), drained, iron flat at moderate heat with an ordinary electric iron, and stored at room temperature, for 24 hr. before use.

Flood (294) recommends using sheets of thick blotting paper which are impregnated by dipping them in sodium aluminate (0.1 to 1 mole/l.), drying, and dipping into saturated sodium bicarbonate. After several days washing with distilled water and drying for several days, the papers are ready for use. They may be used as sheets or cut into strips. Filter paper disks may be impregnated and used in the radial method. It may be mentioned that in washing such papers the most efficient procedure would be to do it chromatographically, using a trough, and downward flow of wash liquid (Chapter VII).

Papers impregnated with infusorial earth, and with charcoal, have been advertised (see Table VII-4), and other impregnating agents such as silica (478) and chromic hydroxide (338) have been reported. Dieckert and Reiser (232) have reported good results in the separation of mono-, di-, and triglycerides using glass-fiber paper impregnated with silicic acid.

VI. PREPARATION OF ADSORBENTS

Adsorbents may have to be *activated*, *deactivated*, or *conditioned* for the particular use and then *standardized* to control the process. These terms probably cover a multitude of different phenomena. Fresh adsorbents may have to be brought to a suitable state, or used adsorbents may have to be cleaned ("recovered") and returned to the desired state. Certain types of treatment and the interpretations of what they do to the adsorbent are given below in an attempt to cover the field in a general way. These are classed under *heat treatments* to bring the adsorbent to a high state of activity, followed by *controlled deactivation*, including the controlled deactivation termed *saturation*; and the treatments designed to remove various *catalytic* or other undesired *chemical* effects of the adsorbent. In each case appropriate standardization is carried out, and this is discussed first.

1. Standardization

The standardization of an adsorbent can be carried out only on the basis of an operational definition of standardization. Neglect of this fact

has led to many difficulties, contradictions, and confusions (385). Recognition of this fact is bringing some progress in the matter. An adsorbent can be standardized on the basis of its capacity *for* some substance or type of substance; but it cannot be standardized on the basis of some concept called "its capacity." An adsorbent can be standardized also on the basis of selectivity *between* pairs (or larger numbers) of substances, but not just on the basis of its "selectivity." The standardization involves a comparison of the adsorbent in question with some other which shows a known degree of standard behavior, or with the defined properties which are accepted as standard. In any case, if a standard is set up, it must be arbitrary to some extent. The alternative is not to set up a standard but to describe the adsorbent in terms related to its function. This gives a means of evaluating the adsorbent, and is in many ways a more profitable procedure than standardization, but it also requires more knowledge of the behavior of the adsorbent, and this, for practical reasons, is often lacking, or even unobtainable.

One of the difficulties met in evaluating adsorbents, as for industrial use, is that they are often used to adsorb substances of unknown chemical nature, such as the "color" of molasses solutions or the "odor" and "taste" of treated water. Since the natures of the substances to be adsorbed are not known, a standard substance has to be taken, such as a "standard molasses solution" in sugar sirup decolorization, against which the adsorbents can be tested. An alternative is to use a "standard reference adsorbent" against which other adsorbents can be tested *via* any molasses solution. A great deal of work has been done to find some pure, easily analyzed substance which can be used as a substitute for these ill-defined "colors" and "odors." Iodine in KI has been widely used, since the brown-colored solutions can be decolorized and the extent of adsorption easily checked by titration with thiosulfate. Hassler (374) states that the iodine adsorption test applied to charcoals seems to be indicative more of the ability of the adsorbent to remove odors and flavors than to remove color bodies. Permanganate solutions have also been used, as have methylene blue or ponceau red solutions. In testing the ability to remove "odor" and "taste" from water, phenol adsorption and adsorption of other pure substances have been investigated. A fundamental, but by no means the only, weakness of these indirect tests is that there is no way of determining the correspondence between per cent of color reduction in, say, molasses solution and the removal of a given per cent of iodine from a stock solution. The tendency in applying adsorbents is more and more to test them on actual samples of the materials with which they are to be used, and to make the tests at a number of concentrations so as to avoid the errors of one-point comparisons (604).

Tests of capacity can be made by break-through experiments or by batchwise adsorption. In the former type the weighed adsorbent is placed as a bed, or column, in a tube, and the test solution is passed in until adsorptive breaks through at the foot of the column. The volume of "empty" liquid up to this point is the "break-through volume." In the latter type of experiment a weighed amount of adsorbent is shaken with a known amount of standard solution and the amount of adsorptive adsorbed is determined from the change in concentration of the solution. As an example of the latter measurement, Miller (639) defines a good grade of charcoal as one 0.25 g. of which will, at equilibrium, adsorb about 50% of the benzoic acid from 100 ml. of a 0.02 *M* solution. (To ensure contact the charcoal is well mixed with the solution and the mixture is evacuated at a water pump to remove occluded gas from the charcoal.)

Another method of testing adsorbents was used by Brockmann and Schodder (118). Technical alumina was activated (dehydrated) by strong heating and then deactivated to controlled degrees by exposure to moist air. An arbitrary set of 5 degrees of activity was set up. The activities were judged by the ability of the given grade to make separations between various pairs of dyestuffs (see below). There was some question, however, about the transferability of data such as these to mixtures of substances other than dyestuffs. Kaufmann (461) suggested that the ability of an adsorbent to separate fatty acids should be tested on fatty acids. Lew, Wolfrom, and Goepp (556), recognizing the arbitrary quality of the choice, set up a standard of good selectivity in work with sugars and derivatives as the ability to separate a mixture of sorbitol (D-glucitol) and D-mannitol.

Weil-Malherbe (978) has suggested that the threshold volume (the break-through volume) per unit weight of adsorbent be used to compare adsorbents and eluents. Claesson (177) has found that the Langmuir equation describes fairly well the adsorption of a number of aliphatic substances on a number of charcoals from absolute ethanol. He writes this equation as:

$$a_i^0 = k_i c_i/(1 + l_i c_i)$$

where a_i^0 is the amount of the ith component adsorbed per gram of adsorbent at the equilibrium concentration c_i, and k and l are constants. In comparing isotherms of homologous fatty acids Claesson observed that this k increases in geometric progression with arithmetic increase in the chain length, giving the relation:

$$k = pq^n$$

where p and q are constants and n is the number of carbon atoms in the fatty acid (no alternation was observed in k). A relation of this type held reasonably well (with different constants) for ethyl esters, dibasic acids, and alcohols, and for five different charcoals. One charcoal showed rather large discrepancies.

Claesson suggested, then, that charcoals can be calibrated and compared for use with homologous series through these constants p and q. A charcoal with a larger q value has greater separating power than one with a smaller q value; a larger p value indicates greater adsorption than a smaller p value. The determination of the p and q values for a charcoal is possible from a single frontal analysis (Chapter IV) of a mixture of two different fatty acids (see the original paper for the calculations), but is more accurately determined from the isotherms of two homologs. Homologs with quite widely different numbers of carbon atoms are taken. The isotherms are measured, and the data plotted as $1/a^0$ against $1/c$ for each homolog. A straight-line plot indicates that the Langmuir isotherm is applicable to the data. The slope of this line is $1/k$ and the intercept on the $1/a^0$ axis is l/k. The constants can thus be obtained for the two homologs. Log k is then plotted against n, and the two points are connected with a straight line, from the slope of which log q can be obtained and log p from the intercept.

LeRosen (541) has made a somewhat different approach to the standardization and evaluation of adsorbents for chromatographic use. He has defined a number of terms the values of which can be used to characterize an adsorbent. These are, for measuring flow characteristics, V_c and T_{50}; for measuring packing in the column, S; and for measuring adsorption affinity, R. These terms are defined as follows: V_c is the velocity of flow of the developing solvent through the column when a constant value is reached. When the solvent is poured on the column the velocity of flow decreases but becomes constant soon after the solvent has reached the bottom of the column. The velocity of flow varied with the pressure difference between the ends of the column, inversely with the length of the column, and was more or less independent of the diameter of the column. The value V_c is measured as the velocity (mm./min.) at which the meniscus of the developer solution moves down the tube. A further investigation of the factors controlling V_c has enabled LeRosen (543), through the concept of the permeability of the column of adsorbent, to calculate V_c for different values of pressure, column length, and solvent viscosity. The relation used is rewritten from the expression for the permeability of a porous solid derived from the Darcy law (673):

$$V_c = k(A/760a)P/\eta L$$

k is the permeability in darcys, A the cross-sectional area of the column in square centimeters; a the interstitial volume of the column in milliliters per millimeter (determined experimentally); P the driving pressure in millimeters of mercury (the difference in pressure between the two ends of the column); η the viscosity of the solvent in centipoises; and L the length of the column in centimeters. Good agreement between calculated and observed V_c values was obtained.

The quantity T_{50} is defined as the time in seconds required for a solvent to penetrate 50 mm. into an initially dry column of adsorbent $9 \times 75 \pm 2$ mm. in dimensions under the full vacuum of a water pump (542). The column is prepared in a standard manner: the adsorbent is poured into the chromatographic tube under the vacuum of the water pump, and settling is aided by tapping the sides of the tube. The surface of the column is leveled without pressing down on the adsorbent. LeRosen found that, when columns of silicic acid were prepared with a filter aid diluent (silicic acid–Celite 535 mixtures), $\log_{10} V_c$ decreased linearly with the volume fraction of silicic acid, and $\log_{10} T_{50}$ increased linearly with the volume fraction of silicic acid. (See Fig. VI-1.)

The quantity S is the length of adsorbent column which will contain unit volume of solvent divided by the length of unfilled adsorption tube required to hold the same volume of solvent (541). The ratio S is therefore a measure of the average packing in the column. LeRosen found S to vary along the column. With the silicic acid-Celite mixtures mentioned in the previous paragraph S increased linearly with the weight fraction of silicic acid (542).

The adsorption affinity **R** is defined as the velocity (mm./min.) of movement of adsorptive zone divided by the velocity of flow of developing solvent (V_c) (541). Thus **R** is the velocity of movement of the zone relative to that of the solvent, and is dimensionless. The measurement of R is made from the lower, sharper edge of the zone. In measurement of changes of zone depth the terms $\mathbf{R}_t$ and $\mathbf{R}_l$ are used for the R values of the trailing and leading edges of the zone (542,545). LeRosen has shown that R is identical with the fraction $T_s/(T_a + T_s)$, where T_s is the average time that an adsorptive molecule spends in solution between each adsorption, and T_a is the average time on the adsorbent (542). With silicic acid–Celite mixtures, $(1-\mathbf{R})/\mathbf{R}$ increases linearly with the volume fraction of silicic acid. The value of R can be calculated with fair agreement with experiment from data obtained from isotherms.

These various quantities, then, can be used to evaluate, characterize, and standardize absorbents. LeRosen (542) suggests that in many cases (but depending on the chromatographic problem) the following ranges of values are satisfactory: V_c = 10 to 50 mm./min.; T_{50} = 20 to 100 sec.; $\mathbf{R}$ = 0.10 to 0.30; and S, no special requirement.

As was referred to above, Brockmann and Schodder took a step, important to chromatographers, when they implemented the concept of standardized adsorbents by setting up a reproducible scale of activities for the versatile adsorbent alumina (118). Since then the concept has been applied in many directions, including the commercial production of standardized adsorbents (see Table VIII-1).

They chose the six azo dyestuffs listed in Table VIII-8 and defined activities of alumina such that under standard conditions the two most

TABLE VIII-8
Grading of Alumina According to the Method of Brockmann and Schodder (118)

	Position of zone		
Grade	Near top of column	Near bottom of column	In the effluent
Highest activity			
I	*p*-Methoxyazobenzene	Azobenzene (not quite at bottom of column)	
II	Methoxyazobenzene (zone 1 cm.from top)		Azobenzene
II	Sudan yellow	Methoxyazobenzene	
III		Sudan yellow	Methoxyazobenzene
III	Sudan red	Sudan yellow	
IV	Sudan red (1 to 2 cm. from top)		Sudan yellow
IV	Aminoazobenzene	Sudan red	
V	Hydroxyazobenzene	Aminoazobenzene	
Lowest activity			

weakly adsorbed dyes were held and separated by the most active alumina (grade I), whereas only the most strongly adsorbed dyes were held and separated by alumina of the lowest activity, grade V. This is shown in the table.

To test the alumina, a chromatography tube, 10 cm. long by 1.5 cm. I.D., is used. Alumina is packed in to a height of 5 cm. and covered with a disk of filter paper. The mixture of two pigments, 2 mg. of each in 2 ml. purified benzene that has been distilled over KOH, and 8 ml. petroleum ether is applied to the column and developed with 20 ml. benzene and petroleum ether 4:1 (v/v). The rate of flow should be about 20 to 30 drops per minute.

Their method of preparing the various grades left something to be desired, since it involved exposure of the higher activity alumina to moist air with occasional testing until the desired activity was reached. This procedure has been improved since it was found that the highest activity alumina could be readily brought to a lower degree of activity

by the addition of an appropriate amount of water which, when mixed into the adsorbent, quickly distributed itself in a uniform manner. Several studies of this kind have been made (88,390,485).

Perhaps the most suitable method for controlling activity (the test method of Brockmann and Schodder, and similar ones still is used to grade the products) was devised and studied by P. B. Müller (663,664). The activity of the adsorbent is measured in terms of the heat (Q) evolved when 50 g. is stirred slowly in a calorimeter with 65 ml. specially purified petroleum ether at 0°C. The most active alumina released 83.5 cal. It could be progressively deactivated (see Fig. VIII-30) by the addi-

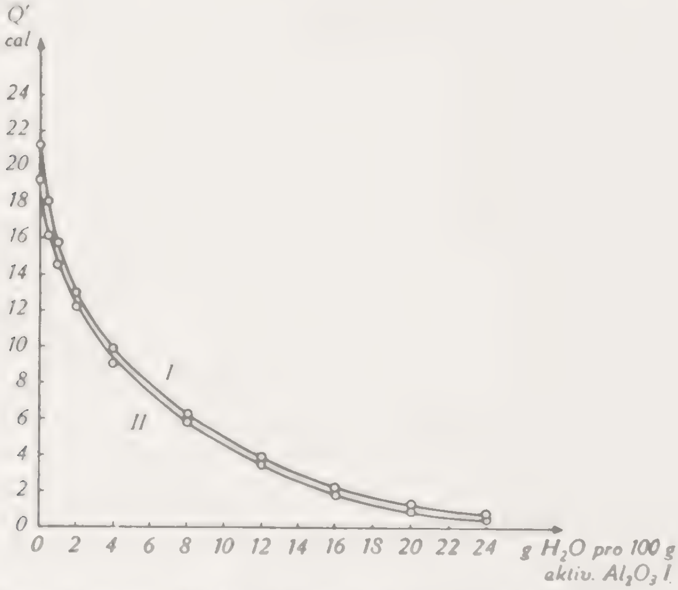


Fig. VIII-3. The activity of the adsorbent is measured in terms of the heat (Q, in calories) that is evolved when 50 g. of adsorbent is mixed with 65 ml. petroleum ether at 0° C. The activity is plotted against the grams of water added to 100 g. of the most active grade of alumina, grade I. (From P. B. Müller.)

tion of water. For a given desired activity a known weight of alumina was placed in a flask, the appropriate quantity of water added, the flask closely stoppered, and the mixture shaken for many hours.

The standardization procedure has been extended to many other adsorbents. For example, Brockmann (113) has obtained bentonite in five grades of activity, silica and silica gel, calcium sulfate, and magnesium oxide, in three, and calcium carbonate in two. The same set of dyes as listed in Table VIII-8 was used, with the same procedure, but the adsorption sequences were not the same with each adsorbent. Changes in sequence were also observed using other solvents, and using adsorbents

such as alumina and silica that had been treated with acid or base. In order to avoid such differences, Brockmann used the set of dyestuffs of decreasing affinity here shown, where R is $C_6H_5NNC_6H_4$: $RNHCOCH_3 >$ $RNHCOOC_2H_5 > R{-}OCOCH_3$ or $O_2NR{-}N(CH_3)_2$ or $pBr{-}R{-}p'{-}N(CH_3)_2 > RCOOCH_3 > RNO_2 > ROCH_3$. These were adsorbed in the same sequence when developed with a variety of solvents and adsorbed on a number of different adsorbents.

It is possible, with the variety of methods available, to prepare quite reproducible and well-characterized adsorbents for any chromatographic use.

Müller (665) utilized the graded adsorbents in an interesting way. To separate vitamin A esters from fish liver oils, he prepared a column containing three grades of alumina: a top layer contained $Q = 50$ grade (see Fig. VIII-3) which held the most strongly adsorbed substances. Below this a $Q = 56.5$ grade held the vitamin A ester, and at the bottom of the column, a $Q = 83.5$ cal. grade removed all the remaining solute from the developer, which, thus cleaned up, could be used again. In another case (666) he used a column of five layers of alumina, with the grades increasing in activity from top to bottom. Columns operating on the same principle but using layers of adsorbents of different strengths, such as calcium hydroxide and alumina, were introduced some time ago (1035). Columns of this type described by Müller have been named "step-graded" columns by Porath (741), who used them as described in Section VIII, 4*C*.

2. Activation

Activation of adsorbents is in general a step for (*a*) the removal of substances (usually but not always water) that block active sites on the surface; or (*b*) the development of improved surface area, or porosity, or surface functional groups, thus to increase the amount of substance that will be taken up per gram or milliliter of adsorbent, or to change its kind.

(*a*). Activation by removal of blocking materials may be done by heating to volatilize the substances, or by washing with inert solvents or chemically reactive reagents which may remove materials that coat the surface of the adsorbent.

Substances like alumina or silica must not be over-heated; otherwise sintering and loss of activity and capacity will occur. Sintering probably produces its effect by smoothing out edges, corners, and other protuberances or by collapsing pores and capillaries. It is the exposed atoms on edges, corners, and other protuberances that are thought by some investigators to be particularly active with respect to similar atoms in a surface (538,893). Irregularly arranged functional groups that may give

rise to "active patches" (915) on the surface may be shuffled into less active groupings (possibly less energetic) under the excessive heating. Such effects would alter activity, or avidity of the adsorbent for adsorptive. Such sintering might also alter selectivity by changing the relationships of active groups or by changing pore sizes in the adsorbent. Thus by shrinking pores to smaller sizes, only smaller molecules would be accommodated, and this would alter the selectivity of the adsorbent—its ability to discriminate between substances (on the basis of size or shape, in this case).

A most convenient way to *activate alumina or silica gel by heating* is to use the simple apparatus shown in Fig. VIII-4. A gentle stream of

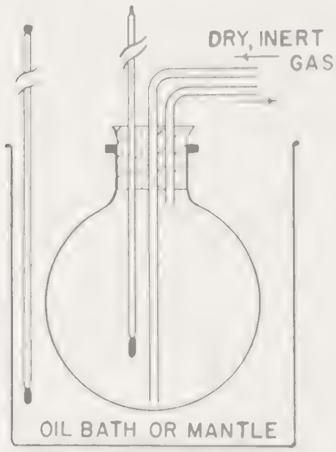


Fig. VIII-4. Apparatus for activating adsorbent. The adsorbent in a round-bottom flask is heated in an oil bath or mantle, and the volatile materials that are released are swept out with a slow stream of dry inert gas. The gas stream is started before heating, and care must be exercised during the warming period because many adsorbents release a good deal of gas (trapped in their pores) on heating.

dried inert gas is led to the bottom of a flask containing the adsorbent which is heated in an oil bath or with a heating mantle. Often, the moisture driven off is seen as vapor at the end of the exit tube, and heating is continued for some time after visible evidence of vapor ceases. Alumina should probably not be heated above 200°C. (643), when it still contains around 8% moisture. The reproducible activation of silica gel was discussed by Bartell and Almy (35). They found maximum activity after heating the gel at 300°C. for 2 hr. Water was still present, but heat sufficient to drive it off caused loss in activity. Presumably the gel structure collapsed. Mair (593) activated silica gel by heating it to

180° to 200°C. in a slow stream of inert gas. This could advantageously be done to the adsorbent in place in the chromatographic tube.

Activation of charcoal, judging from the amount that has been written on the subject (220), is a complicated matter. We consider only certain aspects.

Activation processes for charcoals have been reviewed by many authors besides the few listed here (37,385,793,819). In general, it would appear that high-temperature activation produces a charcoal which may be quite organophilic in its properties, whereas low-temperature activation, particularly in the presence of a strongly oxidizing atmosphere, produces a hydrophilic charcoal which may be made very hydrophilic indeed. In the activation of charcoal *very* high temperatures should be avoided, since above about 1100°C. graphitization, with loss in activity, takes place. Herbst (385) visualizes graphitization as a process in which the adsorption-active carbon molecules in the surface rearrange, mutually saturating each other, so that the charcoal becomes more compact and shows a higher specific gravity. If charcoal is to be used with oxidation-sensitive substances, it may be advisable to heat it to a high temperature and cool it in an inert atmosphere (419).

In some applications charcoals need to be degassed before they show their highest capacity. The solvent, in such cases, seems unable to penetrate the air- or gas-filled capillaries (44). The remedy is to subject the mixture of adsorbent and solution to vacuum (*cautiously*) with gentle boiling, or to evacuate the adsorbent (cautiously at first until most of the gas has been released), and release the vacuum with solvent vapors. This treatment is frequently recommended in directions for decolorization.

A convenient way to activate charcoal for most purposes is to heat it (gently at first) to a red heat in a crucible or quartz beaker in a muffle furnace. The vessel is then transferred to an empty desiccator which is equipped with inlet and outlet gas tubes, and is cooled in an inert atmosphere. The per cent ash content of such a charcoal may be increased because some of the carbon is burned off, but adsorbed oxygen is removed, and the adsorbent may be used with such a sensitive substance as vitamin A (419). Also, presumably, small capillaries and pores are enlarged in the burning process. A good bone charcoal may withstand some 200 kiln-burning revivifications before being discarded (604).

Activation by washing with inert solvents may be used to bring fresh adsorbents to a desired state (542,826,946), as described in connection with silica gels and silicic acid, above; or to recover used adsorbents. This latter is a common practice, and it should be stressed that in general the best and most efficient way to recover spent adsorbent is by a process of washing it with hot solvent in a differential countercurrent manner in a chromatography tube, rather than by a batch process.

TABLE VIII-9
Catalytic Effects of Adsorbents

Reaction	Substance	Adsorbents and references
Acetal formation	Rhodin g trimethyl ester in methanol	Talc (291)
Aminolysis	Amino acids	Charcoal (823,931,964)
Biological inactivation	Alkaloids	Alumina (300)
	Vitamin K	Magnesia and alumina (212)
Catalase activity	In formation of indophenol	Charcoal (840,841)
Colorations	Amines (77 examples)	Bentonites, fuller's earth, kaolin (377)
	Amines and phenol	Clays, silica gel, Permutit, etc. (260)
	Aromatic amines	Acid clays (626)
	In sugar sirups which are being decolorized	Bone charcoal (221)
	Triphenyl methane and other dyestuffs	Polar adsorbents from nonpolar solvents (151,985, 986)
	Vitamin A	Clays & acid earths (619)
Debromination	Lactones	Alumina (798)
	Steroids	Alumina (703)
Decolorizations	Removal of color from pure colored substances	Polar adsorbents (985)
Ether formation	Vitamin A to an ether	Alumina (629)
Hydration	Steroid epoxide to diol	Alumina (394)
Hydration or alcoholation	Vinyl group of porphyrin	Alumina (291)
Hydrolysis	Acyl group from acetylated sugars	Alumina (912)
	Acyl group from triglycerides	Alumina (943)
	Allylic Br by OH in fatty acid, steroid	Alumina (306,896)
Isomerization	Olefins of types BR′C=CH$_2$ and R R′C=CH R″	Silica gel at room temp. (310)
	Steroids	Alumina (850,874)
Olefin formation	Acetic acid split from sterol acetate	Alumina (631)
Oxidation	Fatty acids	Alumina (942)
	Vitamin A to retinine (an alcohol to aldehyde)	MnO_2 (965)
	Vitamin C	Charcoals, some clays (507)
Oxidative fission	β-Carotene, lycopene	MnO_2 (628)
Polymerization, etc.	Acetone to diacetone alcohol (aldol)	Alumina (391)
	Reactions of olefins	Silica gel (593)
Rearrangement	β-γ to α-β unsaturated ketone	Alkaline alumina (770,851)
Saponification	Lactones	Alkaline & neutral alumina (770)
	Sterol benzoates	Alumina (150)

TABLE VIII-9 (*Continued*)

Reaction	Substance	Adsorbents and references
Splitting	Alcohol from synthetic pigments	Alumina (116)
	Picrates of aromatic hydrocarbons	Alumina (736)
	Trinitrobenzoates	Alumina (528)

Note. This table does not by any means cover all possible reactions or all observations of any reaction.

Chemically reactive solvents, such as acids, bases, oxidizing agents, may be used to activate adsorbents by removing adhering materials. This is particularly applicable to the recovery of adsorbents by removal (and recovery) of adhering adsorptive. Also, in the case of aluminas, the nature of the surface can be changed by washing with acid or base, so that different kinds of behavior are shown, as mentioned above. Thus acid-washed alumina converts the adsorbent to an anion exchanger (993), in which form it will adsorb, for example, pantothenic acid (508) and dicarboxylic amino acids (992). Base-washed alumina can adsorb cations (993). Both types of alumina are applicable to inorganic as well as organic ions, but compared with ion exchangers their capacities are quite low. Stannous chloride treatment is recommended to suppress the destruction of autoxidizable substances (486,883).

(*b*). Activation through the development of *improved surface properties* has already been touched upon. Changes in pore sizes can be brought about by heating, as already described. Changes in functional groups on the surface can be produced with charcoals by the heat treatment studied by Bartell and Lloyd (37) described above.

The surfaces of adsorbents have been altered by coating them with metals (772,773), fatty acids, and other substances (4,5,982,983).

3. Deactivation

Deactivation of adsorbents is sometimes necessary either to obtain less curved isotherms, as described above in connection with standardization of adsorbents, or to remove some unwanted property. The latter reason applies to the catalytic properties of some adsorbents which may have deleterious effects on chromatographic analysis.

Some of the *catalytic and other reactions observed with adsorbents* are gathered into Table VIII-9, and others specifically related to ion exchangers are to be found in Chapter IX, Section V. The electron exchange columns of Chapter X would be expected to show their own interesting catalytic properties.

These catalytic properties imply avoidance of certain adsorbents for some uses; avoidance of certain mobile phases with some adsorbents; utilization of certain adsorbents to bring about desired reactions; and the need for special treatments or other means to counter unwanted and deleterious catalytic effects of adsorbents.

Adsorbents should be avoided where, as indicated in the table, they may produce deleterious effects, unless steps are taken to minimize the effects (see below).

Certain mobile phases should be avoided with some adsorbents. Thus acetone and in general carbonyl compounds should probably not be used with alumina or other basic or potentially basic adsorbents because of the tendency for aldol reactions to occur. Oily products from an acetone developer were observed by Dobriner and co-workers (234).

On the other hand, adsorbents are useful in a chemical preparative way; thus acetone can be converted quite efficiently to diacetone alcohol by an alumina column. Also, an alkaline alumina column was used by Sarett (809) to convert a steroid cyanhydrin back to the parent ketone in 90% yield; and an acid-washed column to dehydrate it to the α,β-unsaturated nitrile. One of the most convenient ways of recovering aromatic hydrocarbons from their picrates, styphnates, and trinitrobenzoates, is to pass a solution of the complex through an alumina column (528,736). The picric acid is retained at the top of the column. Further, oxidations can be carried out effectively on a column (628,965).

4. Special Treatments

Special treatments may be given to adsorbents to remove unwanted activities, as described above (and below), when they are to be used in step-graded columns or in gradient elution. Also, the washing of alumina with acid or base falls in this category.

Charcoal may have catalytic properties and a considerable literature about this exists (50,51,374,391,680,823,929,967,968,1024,1038). The deaminating property of some charcoal may be eliminated by treating the charcoal with potassium cyanide (as described above) or with H_2S, and the oxidizing behavior can be eliminated by heating red hot and cooling in nitrogen, as described.

The fact that some adsorbents may have deleterious effects on certain substances is not a serious limitation on chromatography, since usually suitable adsorbents can be found that are not reactive. Thus Aebi and Reichstein (9) chromatographed certain acetylated glucosides on magnesium silicate instead of alumina, for the former did not cause loss of acetyl groups.

"Specific Adsorbents" have been reported by Dickey (231,231a). He prepared silica gel in the presence of a given substance, e.g., methyl orange or ethyl orange, and then removed the substance. The gel then showed an ability to adsorb the substance in the presence of which it was prepared, in preference to related substances. This was laid to an effect of the substance in acting as a template during the formation of the gel structure. When extracted the molecules of the substance then left holes of the right size and shape specifically to pick up chiefly other molecules of this size and shape. The specific structure seemed not too stable (66). Specific adsorbents, "tailor-made" from sodium silicate by Curti and Colombo (208), have been used to separate stereoisomers of camphorsulfonic and mandelic acids; enrichments of 30% and 10%, respectively, of one of the isomers were obtained.

5. Relative Strengths

Many tables have been set up purporting to show the *relative strengths of adsorbents*. These may be divided into two types: gradations based on *differences in activity* of given adsorbents; gradations based on *differences in functionality* of the surface. The former have been discussed in connection with activation of adsorbents, and are produced by prewashing, or by graded deactivation, or by special activation. The activity and specificity of the surface can be changed by the activation process, especially with charcoal, upon which a great deal of work has been done. As an example, Bartell and Lloyd (37) were able, by high-temperature activation, to produce an organophilic surface on purified charcoal. With low-temperature activation in a highly oxidizing atmosphere a surface was produced which the investigators said approached in adsorptive properties, the hydrophilic silica. The properties of the surface of amphoteric adsorbents (such as alumina) can be changed by prewashing. The functionality can also be changed by coating the adsorbent surface.

Gradations in functionality can be obtained by the use of different adsorbents. Strain (888) has ranked adsorbents on the basis of increasing strength (Table VIII-10), with, of course, an awareness of the contingency of the relations. Sometimes a group of adsorbents may show similar selectivity. The choice of one of them then rests upon other considerations. For example, Lew, Wolfrom, and Goepp (556) report that in the separation of sugars and derivatives seven clay adsorbents which were tried showed about equal adsorptive selectivity on the basis of an arbitrary requirement: the ability to separate a mixture of sorbitol and D-mannitol. One of those of intermediate adsorptive strength was chosen for standard use.

Excellent examples of differences in functionality of the surface are provided by exchange adsorbents (Chapter IX). Resins are available with only one type of exchanging group in the surface. It is then easy to rank such substances: among the cation exchangers, the carboxylic vs. sulfonic functional group resins; among the "acid adsorbers," the amine vs. quaternary ammonium resins.

TABLE VIII-10
Graded Series of Adsorbents
(Strength of adsorption increases down the table)

Strain (888)	Hesse[a] (389)
Sucrose, starch	Calcium oxide
Inulin	Calcium fluoride
Magnesium citrate	Alumina (Merck)
Talc	Sugar charcoal
Sodium carbonate	Zinc sulfide
Potassium carbonate	Chromium trioxide
Calcium carbonate	Basic alumina
Calcium phosphate	Acid alumina
Magnesium carbonate	Floridin
Magnesia (Merck)	Franconite
Lime (freshly and partially slaked)	Activated charcoal
Activated silicic acid	
Activated magnesium silicates	
Activated alumina, charcoal, magnesia (Micron brand)	
Fuller's earth	

[a] Dyestuffs were used in the ranking.
Note: The order depends to some extent on the solvents used.

VII. MOBILE PHASES

1. Definitions

Mobile phases used in adsorption chromatography are classed as solvents, developers, eluents, or displacers depending on their functions. *Displacers* are strongly adsorbed substances used pure or, more usually, dissolved in a solvent or a developer, that replace the adsorptive on the adsorbent, expelling it from the stationary into the mobile phase. An example is given in Section VIII. *Eluents* remove the adsorptive from the adsorbent. This is their function. If their eluting power is high, they may be classed as displacers especially when used not pure but diluted with a solvent; if it is low, they verge on developers. Eluents are best chosen to be readily separable from the materials eluted. Thus their boiling points should not be close to the zone materials that are to be

recovered, and preferably they should be chemically different. Thus to elute neutral stable materials, acid or basic substances might serve, and later be readily separable from them, and vice versa. *Developers* are less strongly adsorbed than eluters, and like them may be pure liquids or mixtures. They cause zones to move by competing favorably with the adsorptive for the stationary phase (and with the stationary phase for the adsorptive). Thus developers may be substances that are somewhat more strongly adsorbed than the zone materials, and they may be superior solvents for the zone materials. The choice of developers is discussed in Section IX, and in a general way in Chapters XIII and XIV. *Solvents* are, in general, more or less inert toward adsorbent and adsorptive. They are conveniently used to bring the mixture that is to be separated on to the column. As they become less inert toward the adsorbent they take on the character of developers.

2. Choice of Developer

There is one fairly good rule which can be applied in the choice of a developer. This is to choose a substance in which the components of the mixture are not too soluble. This is usually a liquid with a low molecular weight. To go with this one chooses an adsorbent that adsorbs the developer somewhat, but not too strongly. If the developer is adsorbed to some extent it helps to ensure that the components of the mixture to be adsorbed will not be too firmly bound. Fine differences in chromatographic effectiveness may be achieved by the use of a given adsorbent with given developers which are members of an homologous series, or which are corresponding compounds, or solutions of graded concentration.

If a developer turns out in use to be not satisfactory one can either change to another developer or else modify the one in hand. Modification usually involves addition to the liquid of some substance which acts to expedite (or to delay) development. A frequent need is for more rapid development of the chromatogram, which can be obtained by adding to the developer a more strongly adsorbed substance, either preparing all of the developer of the same concentration or else adding the more strongly adsorbed substance in *gradually increasing* amounts so that the displacing effect of the developer steadily rises. The intention here is to force the segregation of zones in the column by gradually increasing the eluting power of the developer. One may thereby cause the zones to march consecutively out of the column. This method is in fairly common use. Sometimes the order of already partially separated zones may be changed by a change in developer, which may cause some confusion even though eventual good separation with a different order of zones may be obtained

(255,541,826,887,892). Strain has found many striking examples of this effect (889). An example of the use of developer of graded strength is given in Section VIII.

Eluents (which also serve as developers) may be ranked in the order of their effectiveness in removing some adsorptive from a given adsorbent. Such ranking can be derived from Section IX. In many cases the ranking would change with the nature of the adsorptive, and usually would change to some extent with change in adsorbent. Fortunately, however, it has been possible to set up rankings of eluents that hold fairly well over classes of adsorbents. With such series the eluent effect for a given type of adsorptive begins in some region along the series; eluents beyond that point are effective desorbents; substances up to that point are not very effective.

The gradation in behavior of a series of eluents (*1*) may depend upon the differences between homologs; (*2*) it may depend upon concentration differences; (*3*) it may depend upon differences in functionality. The differences in the first two cases are largely differences in degree; in the last case they are differences in kind. The most marked differences in eluent activity can be found in series graded because of differences in functional type. Another gradation can be produced by change in temperature. Since adsorption decreases with increase in temperature, then usually the eluent power of a solution would be increased by using it hot.

Class 1—gradations of eluents based on differences between homologs. Because of convergence in homologous series the members near the beginning of the series show greater differences among themselves than higher members. The first few members, particularly the first member, may show exaggerated differences in properties. Rossini (791) pointed out that the introduction of a CH_2 group does not produce a constant increment in the heat of combustion of normal paraffins and primary alcohols until the number of carbon atoms exceeds five.

When they are compared against benzene as the adsorptive and silica gel as the adsorbent the alcohols fall in the series of increasing strength of adsorption (38): isoamyl, *n*-butyl, *n*-propyl, ethyl, methyl (see Fig. VIII-5). The same order of strength of adsorption is observed against purified charcoal (38) (Fig. VIII-6), but the benzene is more strongly adsorbed than the alcohols. Further examples of eluent behaviors of homologs are contained in the next section. In general, the behavior as eluents will follow the homologous series; the direction, whether up or down the series, is considered below.

Class 2—gradations of eluents based on differences in concentration. The effect of admixture on eluting power can be derived from the discussion in Section IX. With water-alcohol mixture the eluting effect increased with

the per cent of alcohol, markedly for acetic, tartaric, succinic, and oxalic acids, less so for benzoic and salicylic acids on charcoal (Fig. VIII-7). In general, it may be said that admixture can improve the eluting ability of

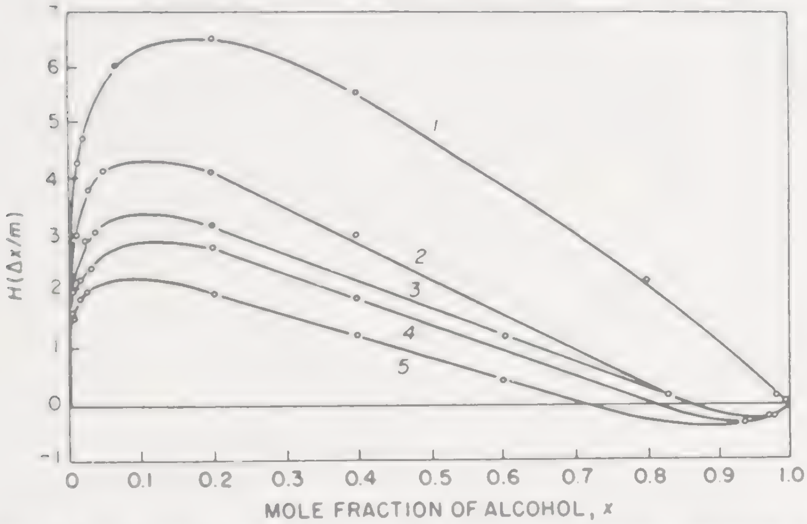


Fig. VIII-5. Adsorption of the alcohol by silica from binary mixtures of various alcohols with benzene over the entire concentration range. H = total number of millimoles in solution; Δx = mole fraction change due to absorption; m = weight of adsorbent in grams; 1, methyl alcohol; 2, ethyl alcohol; 3, n-propyl alcohol; 4, n-butyl alcohol; 5, isoamyl alcohol. (From Bartell and Scheffler (38).)

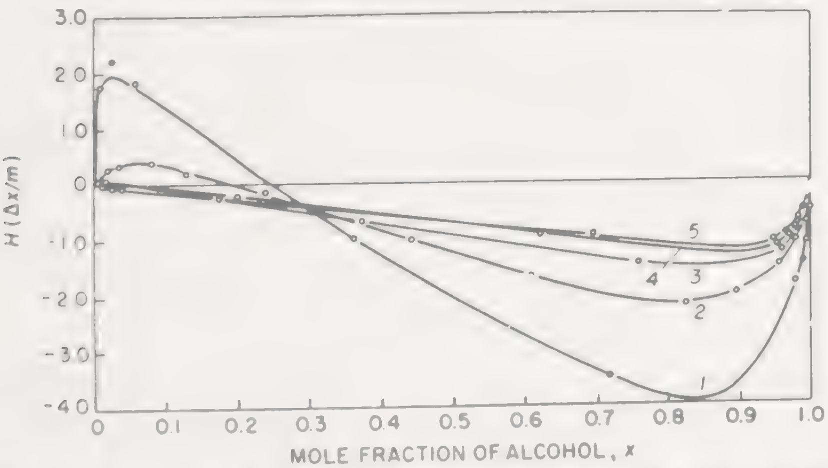


Fig. VIII-6. Adsorption of the alcohol by purified ash-free blood charcoal from binary mixtures of various alcohols with benzene over the entire concentration range. See Fig. VIII-5 for explanation of symbols. (From Bartell and Scheffler (38).)

an eluent. This would seem to follow from the observation of Heymann and Boye (395) that adsorption of a substance from a pure solvent is almost always lowered upon addition of a second solvent. However, Mose-

ley, LeRosen, and Carlton (657) do not find minima in the curves of R against per cent of second or third component in mixed developers. The effects of admixture need further study.

Class 3.—gradations of eluents based on differences in functionality. In this classification belong many of the series of eluents found in the literature. Trappe (942) set up such a series on the basis of heats of wetting and other properties. These relations are shown in Table VIII-11. He named this the "eluotropic series." A somewhat different series is

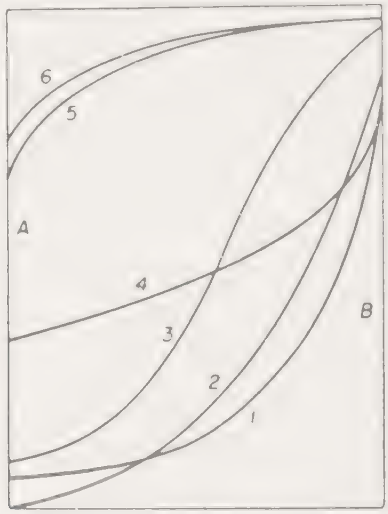


Fig. VIII-7. Adsorption of acids from water–alcohol mixtures. (A = 100% alcohol, B = 100% water.) The mixture was initially 0.025 N with respect to the acid. Adsorption (on 2 g. charcoal from 25 ml. solution) is plotted on the ordinate; the composition of the solvent is plotted on the abscissa. 1, Acetic acid; 2, tartaric acid; 3, succinic acid; 4, oxalic acid; 5, benzoic acid; 6, salicylic acid. (From Schilow and Pewsner (818).)

used by Reichstein for selective elution of mixtures from alumina columns (274,686). A series derived from some of his work is shown in Table VIII-12. Essentially the same series is used by Ruzicka (449). It is evident that these two series do not agree in detail. Strain (888) has given a table of eluents which is more extensive than the previous two (see Table VIII-13). It is recognized that all these series are subject to some variation depending upon the type of adsorbent and the nature of the adsorbed compounds. The use of a series in an analysis is illustrated in the following section.

The direction of movement of the mobile phase can be downward or upward through the column (or radially in radial chromatography). It is not immaterial what the direction is, because if in downward development a denser zone follows a less dense the former will encroach on the latter

TABLE VIII-11
Trappe Eluotropic Series[a] (42)
(Eluting power increases in numerical order)

1. Petroleum ether	6. Benzene	11. Acetone
2. Cyclohexane	7. Dichloroethylene	12. *n*-Propyl alcohol
3. Carbon tetrachloride	8. Chloroform	13. Ethyl alcohol
4. Trichloroethylene	9. Ether	14. Methyl alcohol
5. Toluene	10. Ethyl acetate	15. Water

[a] Eluting power against polar solids.

TABLE VIII-12
Graded Series of Eluents Taken from the Papers of Reichstein and Co-Workers[a] (274)
(Eluting power increases in numerical order)

1. Petroleum ether	5. Methanol
2. Benzene (anhydrous)	6. Ethyl acetate
3. Ether (absolute)	7. (Glacial acetic acid)
4. Chloroform	

[a] Eluting power against Brockmann alumina.

TABLE VIII-13
Strain Graded Series of Eluents[a] (888)
(Eluting power increases in numerical order)

1. Petroleum ether 30–50°	11. Esters of organic acids
2. Petroleum ether 50–70°	12. 1,2-Dichloroethane, chloroform, dichloromethane
3. Petroleum ether 70–100°	13. Alcohols
4. Carbon tetrachloride	14. Water
5. Cyclohexane	15. Pyridine
6. Carbon disulfide	16. Organic acids
7. Ether (absolute)	17. Mixtures of acids and bases with water, alcohol, or pyridine
8. Acetone (absolute)	
9. Benzene	
10. Toluene	

[a] Eluting power against polar solids.

in the mobile phase and possibly blur the front. Thus, in general, for sharpest fronts when separating homologs where the lower molecular weight ones appear in the effluent first it would appear that the developer should be run upwards. The effect of velocity of flow of developer was discussed in Section III, above.

A convenient way of characterizing the progress of a development or an elution is in terms of the volume V of solution required to fill the bed of adsorbent.

VIII. PROCEDURES

Examples of different kinds of chromatography, with mention of batch-wise adsorption, are given in this section.

TABLE VIII-14

Representative Surface Area and Density of Some Adsorbents

Material	Surface area (m.²/g.)	Gas (°C.)	Bulk density (g./ml.)	References
	Carbonaceous Adsorbents			
Bone char, new	120	$N_2(-195°)$	0.8	(223,679)
Coconut-shell charcoal	1700	"	—	(223)
Columbia carbon	1397	"	—	(245)
Grade L	1152		0.48–0.56	
Darco G-60	1300	$N_2(-195°)$	—	(223)
S-51	500	"	—	(223)
Graphite, powdered	30.73	$N_2(-195°)$	—	(267,679)
Norit	930	"	—	(223,679)
A	935	N_2	—	
B	850	"	—	
F	745	"	—	
SG II Extra	1040	"	—	
PC III	825	"	0.385	
RHS I	1140	"	0.48	
Pittsburgh activated carbon				
BPL	1050–1150	N_2	0.5	
SGL	950–1050	"	0.48	
OL	1000–1100	"	0.44	
CAL	1000–1100	"	0.44	
Polymer carbon	470–1400			(1012)
Suchar	850	"	—	(223)
Zeo-Karb	0.2	"	—	(679)
	Inorganic Adsorbents			
Activated alumina				
F-20	210		1.09	
XF-21	150		1.07	
H-51	350		0.95	
Attapulgus clays				
Attaclay	125		0.44–0.5	
Attasol	125		0.53–0.58	
Attasorb	125		0.19–0.24	
Bauxites	82–289	$N_2(-195°)$	1.30–0.86	(380,514)
Bentonite ($<0.3\mu$)	18.7	"	—	(597)
Diatomaceous earth	<1–4.2	"	0.29–0.32	(245)
Filter-Cel	16			
Super-Cel	5.0			
Hyflo Super-Cel	2.0			
Celite 503	1.5			
Celite 545	1.0			
Filtrol Grade 13 clay	300		0.72	
Florex	128		0.5–0.56	
Florisil	298		0.48–0.51	
Florida fuller's earth	120		—	
Fuller's earth	129	"	—	(223)
Glass, porous (Corning)	120	"	—	(268)
Kieselguhr	22.2	$N_2(-195°)$	—	(453)

TABLE VIII-14 (*Continued*)

Material	Surface area (m.²/g.)	Gas(°C.)	Bulk density (g./ml.)	References
	Inorganic Adsorbents (continued)			
Permagel	150		0.72–0.83	
Pumice	0.38	"	—	(130)
Quartz, 400 mesh	0.361	"	—	(312)
Silica gel				
Davison 912	832		0.64–0.75	
922	832		0.64–0.75	
963	832		0.48–0.56	
Silica aerogel V	410	CCl_4	—	(480)
Vermiculite	0.52	$N_2(-195°)$	—	(370)
Vermiculite exfoliated	10.35	"	—	(370)

Note. Many of these values are from Deitz (222), who gives a much more extensive table. See also his Bibliography of Solid Adsorbents, 1900 to 1942 (220) and 1943 to 1953 (220a). Other data are from industrial literature. Surface areas and other characteristics of commercial adsorbents may change from time to time. The data in this table indicate at least orders of magnitude of the various values. Addresses of the producers may be found in the appropriate Appendix.

1. Batchwise Adsorption and Decolorization

The practical application of adsorbents is an old art (220,576). One of the most common uses of adsorption is for purification of gases or liquids. In such applications the objective often is to remove moisture, "color," "odor," or, in general, undesirable components of the mixture without affecting appreciably the desirable components. In another type of application, which may also be called purification, the object is to collect and concentrate one or more components of the mixture, leaving behind the others. These two uses may be combined: one fraction of the mixture is left in the purified solution; the other is recovered as an adsorbate (adsorbent + adsorptive).

A number of aspects of adsorbents and their uses will be omitted here. There will be no discussion of methods of manufacture of adsorbents (462,496,534,604,981) or of special industrial applications, though some will be listed. There will be no discussion of the application of adsorbents in such uses as air conditioning (534,604), dehydration (534,604), gas masks (534,604), solvent recovery (534,604), petroleum refining (355), sugar manufacture (374), water purification (53), catalysts, as carriers of insecticides or other agents, in medicinal applications, or in connection with adhesives, binding agents, etc.

Adsorbents are judged on characteristics that include surface area. A large surface area can be provided by using a finely divided adsorbent. It can also be obtained by using a very porous substance. Quite large surface areas are shown by substances which are both very porous and finely divided (223,222). Table VIII-14 shows the surface areas of a

number of adsorbents. But from the discussion in the previous chapters it must be concluded that a "large surface area" is not the sole requirement for a useful adsorbent. There is needed a large surface area *accessible* to the adsorptive in question as well as a preferential ability to take up the adsorptive. These requirements are important in the evaluation of adsorbents.

It is essential not only to obtain contact between the mobile phase and the adsorbent surface but also to permit sufficient duration of contact. The requirement of contact thus has two aspects: good contact, which provides that molecules of adsorptive need diffuse over only a short path to reach a surface; and duration of contact, which allows time for the adsorption reaction itself. Swearingen and Dickinson (901) studied the rates of adsorption of mono-, di-, and trichloroacetic acids by silica gel and blood charcoal. The rates of adsorption and the time required for reaching equilibrium were found to depend on the amount of agitation, the nature of the adsorptive, the concentration of the adsorptive, and the nature of the adsorbent.

Maximum adsorption occurs at the equilibrium point, but this may not be a useful goal for any or all of several reasons. It may be impractical to wait for equilibrium in those cases where this state is approached slowly. For example, in the exchange adsorption of cupric ion from aqueous solutions on 20- to 30-mesh carbonaceous zeolite (Zeo-Karb-M) it was found by Beaton and Furnas (57) that most of the exchange had occurred in 6 to 8 hr. In 6 hr. 1.8 meq. were taken up per gram of zeolite. But adsorption continued slowly, so that at the end of 7 days the adsorption had increased to 1.9 meq./g. This may be an example of a problem in contact (46), the internal surface of the exchanger being somewhat less accessible to the ion than the outer surface of the particles.

Again, the attainment of equilibrium may be undesirable in cases where substances to be separated show markedly different *rates* of adsorption. Dubinin (250) noticed, when using fine-pored charcoal, that the appearance of reversal of the adsorption series with the acids propionic, valeric, and heptylic was increased when experiments were carried out using durations of contact insufficient for reaching equilibrium.

Adsorbents are used not only for removing molecularly dispersed substances from solution but also for removing colloidally dispersed substances (373,374), as well as suspended matter of even larger particle size. Soaps may be adsorbed from suspension by some charcoals. Use of this is made in the removal of free acids from edible oils. The free acid is neutralized and the soap is adsorbed. This allows complete removal of the acid which otherwise, owing to hydrolysis, would be distributed between the oil and caustic phases (373,374).

It appears that some of these applications rest upon the fact that many adsorbents in aqueous or other ionizing solutions take on a characteristic charge (Table VIII-15). Where this charge is opposite to that of the colloidal micelles or other particles, the adsorbent can effectively bring about flocculation and removal of these substances. The field does not seem to have been reported very thoroughly. Hassler (373,374) states that whereas lyophobic colloids such as gold sols are removed by activated charcoal the effect appears to be due to salts in the adsorbent because an ash-free charcoal is without effect. This may be, however, a matter of the charge on the different charcoals. He states further that lyophilic

TABLE VIII-15

Characteristic Charges Taken on by Some Particles in Water Suspension

Particles usually negatively charged	Particles usually positively charged
Clays: bentonites, montmorillonites	Alumina, basic hydroxides
Insoluble acids: tungstic, stannic	Sols of metal oxides, ferric oxide
Acid dyestuffs	Basic dyestuffs
Soap micelles	Invert soaps, "onium" soaps
Silica, glass	
Sols of metals, metallic sulfides	
Agar-agar	
Proteins in basic solution	Proteins in acid solution
Most charcoals[a]	
Cotton, paper, mastic	

[a] Purified sugar charcoal activated near 1000°C. gave both positively and negatively charged particles; when activated at temperatures near 400°C., or in a highly oxidizing atmosphere below 150°C. it gave only negatively charged particles (37). For bibliography, see V. R. Deitz (220,220a).

colloids such as gelatin and gums are well adsorbed by some charcoals. Some chars (charcoals) when suspended in water act as negatively charged lyophobic substances in that they can be coagulated by cations, especially if the cations are strongly adsorbed.

Mantell (604) states that the majority of colored materials encountered in industry are negatively charged, and that ordinarily charcoals will decolorize such substances more efficiently the higher the acidity of the solution; oppositely charged substances, then, would be best adsorbed in alkaline solution, and amphoteric substances close to their isoelectric points. The adsorption of uncharged materials is less affected by acidity or alkalinity. The problems are, however, rather complex, so that it is difficult to lay down very general rules.

There is a large literature on the ability of adsorbents to adsorb ions (37,249,299,604,640,793). Thus, charcoals can be produced which are negatively or positively charged in aqueous suspension or not charged at all. Alumina can be prepared by suitable washing treatments to act as

anion or as a cation exchanger (836,837). For example, if alumina is treated with acid it takes on anion exchange properties; if the acid was HCl the adsorbent becomes capable of exchanging Cl^- ions for others (see above) (508).

The ability of adsorbents to take up charged particles sometimes becomes an inconvenience. Under some conditions the adsorbent, especially if it is finely divided, becomes peptized and is then difficult to remove from suspension. This is sometimes noticed with decolorizing charcoal, often in alcohol solutions. The charcoal becomes so well peptized that it passes through filter paper and even resists centrifugation. The remedy in this case is to stir into the suspension a small amount of filter paper pulp. This rapidly collects the charcoal particles, and the whole mass can then be filtered off. In other cases a finely divided powder of opposite charge, or a polyvalent ion, is stirred into the suspension until the particles are flocculated. An alternative procedure is to carry out the adsorption process (or decolorization) chromatographically.

Adsorbents are able to take up relatively large particles. For example, bacteria can be taken up on many adsorbents (58,523,694). Ore flotation phenomena probably also belong in this classification.

In industrial as in laboratory practice decolorization is often carried out by adsorption as a purification step preparatory to the isolation of a desired product. One of the largest uses of charcoal is in decolorizing sugar sirup as a step in preparing pure crystals (220). Another large use is in the water purification industry (53,374). Extremely large quantities of fuller's earth and other adsorbents are used in purifying petroleum products (604). In the laboratory adsorption is used for similar purposes: to remove impurities, especially those which may inhibit crystallization or produce foaming, and to produce a clear liquid preparatory to distillation or other manipulations.

The directions given in laboratory procedure for bringing about decolorization often call for the use of concentrated solutions and heat—sometimes even several hours of refluxing. Insofar as the decolorization proceeds by an ordinary adsorption process such directions are not necessarily realistic, even though conventional. Adsorption is usually fairly rapid, and it usually decreases with increase in temperature (except chemisorption, which may increase). The reasons sometimes given for the use of heat and long contacting are that adsorption is greater from more concentrated solutions, which in many cases can only be obtained in hot solvents; also that in many cases the purified product can be crystallized directly upon cooling the solution after removal of the adsorbent. The duration of contacting is justified by the recognition that adsorption reaches a maximum at equilibrium. It is often an advantage to

be able to crystallize directly after decolorization; however, this and the other advantages are very often offset by the fact that in using a more concentrated solution both the product and the undesired adsorptive are concentrated, so that the adsorption of both may be improved. Furthermore, although the extent of adsorption increases (to the point of saturation of the adsorbent) with increase in concentration, the increase is relatively less at the higher concentrations. This effect is more noticeable the more curved is the isotherm, so that the advantage gained may even be negligible. Also, if the colored impurities are strongly adsorbed and the desired product weakly, so that the isotherm of the former is strongly curved compared with that of the latter, then adsorption of product may be improved by increased concentration relative to adsorption of impurities.

The most questionable feature of the conventional procedure is, however, the long heat treatment. Decolorizing agents are not to be thought of as chemically inert. There is an extensive literature on the catalytic properties of charcoal and other adsorbents (50,51,374,680,823,967,968, 1024,1038). Some of the reactions are gathered into Table VIII-9. Many of these reactions do not require heat.

It is quite likely that many decolorizations are not purifications in the sense that they might at first appear to be. Some charcoals are known to possess strong reducing action—possibly because of adsorbed carbon monoxide. Others are actually oxidizing agents, perhaps through the agency of adsorbed oxygen. Decolorization of unsaturated substances may be produced by reduction or by oxidation to smaller fragments. The reactions would probably be aided by heating. Loss of color to a solution in the presence of charcoal, then, does not necessarily imply adsorption.

Usually, decolorization can be effected by use of a suitable solvent and adsorbent, using as a guide some of the general principles derived in this book. For example, in a Bucherer hydantoin synthesis involving an oleyl side chain the reaction product was dark. Partial decolorization was obtained by overnight boiling of the mixture in alcoholic solution with charcoal (the conventional procedure). Even more prolonged treatment did not produce further decolorization and the product did not crystallize well from the mixture. The alcohol was removed and a dilute solution in ether was prepared. This was passed cold through a small amount of charcoal held on a wad of cotton in the stem of a funnel (a chromatographic procedure). Complete decolorization was quickly obtained. In this case the alcohol was not only a good solvent for the reaction products but was also probably well adsorbed, thus competing effectively with the colored substances for the surface of the charcoal.

In general, then, *if trouble is experienced in obtaining decolorization, the procedure should be tried out in the cold, using a solvent which is not too well adsorbed, and using a relatively small amount of adsorbent to which the mixture is applied in a countercurrent manner.*

2. Frontal Analysis

In all column adsorption methods, the chromatography tube must be packed with adsorbent so as to give as homogeneous a column as possible. Manipulations are described in Chapter VI, Section VI. An example of the frontal analysis procedure is taken from Claesson's work (177).

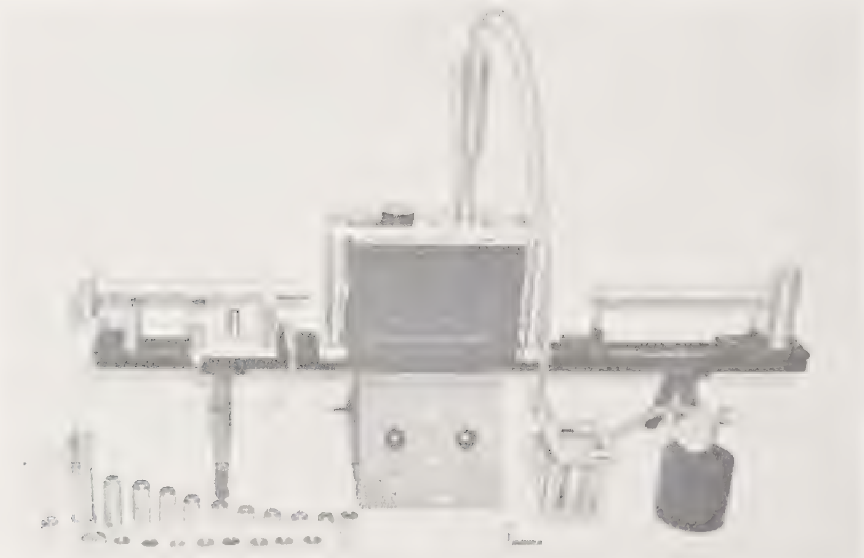


Fig. VIII-8. Modified Tiselius-Claesson adsorption analysis apparatus. Gas pressure from the reservoir in the right foreground drives a syringe in the metal housing above the metal column, which extends into the thermostated bath at the center of the picture. Sections of disassembled column are shown in the left foreground. The effluent from the column passes through a cuvette inside the bath, and is examined interferometrically by means of the optical system on right and left of the bath. (From R. T. Holman, private communication.)

Tiselius' and Claesson's chromatography device (177,935) consists essentially of a syringe through which pressure can be applied to the solution to be analyzed, forcing it through adsorbent that is held in connected metal tubes or "filters." The effluent passes through a narrow channel and through a cell where the refractive index is determined interferometrically and thence to the collection vessels. An apparatus of this type is illustrated in Fig. VIII-8 (418). See Appendix for sources.

The "filters" that are to hold the adsorbent are weighed, packed with

adsorbent, and weighed again. The weight of adsorbent should be close to a "normal" value (within 5%); otherwise the packing is considered irregular, and should be redone. The usual procedure is to construct a column out of several of these packed filters, joined together by couplings. The couplings, designed by Hagdahl (357), have capillary bores within which the effluent from one filter is thoroughly mixed before it passes to the next filter. This mixing tends to iron out irregularities in the front, as shown schematically in Fig. VIII-1. As shown in that figure, the front sharpening is improved by the use of a narrower diameter column below the wider one, an arrangement utilized also by Mair, and one that is widely applicable in column chromatography (593,596).

The column of adsorbent in the apparatus is washed with the solvent to be later used as carrier for the mixture to be analyzed, until all air is displaced; then the mixture to be analyzed, in solution, is passed into the column, with care to avoid getting gas bubbles into the system.

As effluent appears, it is analyzed for solute. The first liquid that appears comprises that in the channels of the apparatus (if any) leading to the column, and that in the interstices of the bed of adsorbent. After this (previously determined) amount of liquid has issued, the measurement of the retention volume can begin. This is the volume of liquid from which adsorptive is removed by the adsorbent up until the column is saturated by that component, which then "breaks through" the bed and produces a "front" or sudden change in concentration.

Examples of the use of the method have been given by Claesson and others (177). One such is shown in Table VIII-16 and Fig. VIII-9 (177).

TABLE VIII-16
Frontal Analysis of a Mixture of Fatty Acids[a]

Mixture	Concentrations (relative %) As prepared	As found
Octanoic acid	20	21
Decanoic acid	20	19
Tetradecanoic acid	20	20
Hexadecanoic acid	40	41

[a] From S. Claesson (177).
Note: The solvent was absolute ethyl alcohol, and the adsorbent Carboraffin CIV.

The analysis is of a mixture of four fatty acids. The method for calculating composition from the data when more than two components are present in the mixture is complicated, and must be sought in Claesson's original paper.

3. Development Analysis

Examples of development analyses have already been given, for example, with dyestuffs, in the standardization procedure of Brockmann, Section IV,

above. With colored adsorptives, an experiment very similar to one of those performed by Tswett (947) can be carried out in the following way (163,167). A small amount of fresh grass or a carrot leaf is cut up and rubbed up in a mortar with a few milliliters of 95% alcohol and about twice as much petroleum ether. The solvent mixture becomes quite green with the leaf pigments. Acetone may be used instead of the alcohol, but one or the other polar solvent is necessary to help extract the pigments. The polar solvent must be completely removed before adsorption. The green solvent mixture is decanted from the vegetable residue into a separatory funnel and washed with water by swirling the mixture (to avoid emulsion). A milky water layer forms and is drawn off. The washing

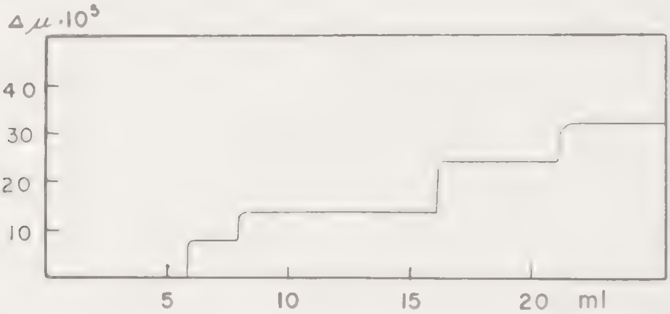


Fig. VIII-9. Frontal analysis of the mixture of fatty acids shown in Table VIII-16. (From Claesson (177).)

is continued until the water layer remains clear. The washed petroleum ether layer is separated and about 1 ml. is set aside for later use. The green petroleum ether layer is now carefully dried by swirling in a flask with a little anhydrous sodium sulfate. If all the materials are on hand this whole operation, from green grass to dried green extract, should take less than an hour.

While the pigment is drying, a column of adsorbent is prepared. The glass tube of a medicine dropper, or a 15-cm. piece of 7-mm. tubing drawn down slightly at one end, is taken, and a small wad of cotton is placed lightly in the constriction. A little calcium carbonate, U.S.P., or confectioner's sugar is put into the tube and gently packed on the cotton with a flat-end glass rod or wooden stick. Adsorbent is added in small portions and gently pressed down until a column 2.5 to 4 cm. long has been formed. Now the dried leaf extract is dripped into the tube and allowed to run evenly into the adsorbent until a green zone 3 to 4 mm. deep is formed, ahead of which colorless solution has run. If the petroleum ether extract was not washed sufficiently or was not dried, the zone does not form and the green solution runs on through the tube. The green zone is

developed with a few milliliters of solvent. If the adsorbent is calcium carbonate, use benzene as the developer; if sugar, use petroleum ether solution containing $^1/_3$ to $^1/_4$ part of benzene.

The development is fairly rapid. As the developer passes into the adsorbent the green zone spreads and becomes differentiated into an upper thin dark-green zone, a lower blue-green zone, an orange zone below this, and a fast running yellow zone still lower. The upper green zone is chlorophyll b, the blue-green zone is chlorophyll a, the orange zone contains xanthophylls, and the yellow zone carotenes. The zone of carotenes may easily be washed out of the column. The other zones can be recovered by extruding the column, cutting it between the zones, and eluting each in a separate tube or in a test tube with petroleum ether or benzene containing a drop of alcohol. The separation of individual carotenes or xanthophylls requires more subtle chromatography. This experiment can easily be scaled up. The undried petroleum ether extract, set aside above, can be used with a fresh column of adsorbent to see the effect of traces of polar material on the chromatography. Or, a *trace* of alcohol can be added to some of the dry petroleum ether extract and this chromatographed.

As examples of development where the components are colorless, experiments described by Wolfrom and Binkley (76), with sugars and sugar derivatives, serve. The adsorbent is Florex xxx (see Section V, above). To a column of adsorbent 6 cm. long in a 0.9-cm. I.D. tube is applied 0.5 ml. of 95% ethanol solution containing 1 mg. sorbitol (from the reduction of glucose) and 0.9 mg. glucose. The chromatogram is developed with 4 ml. 95% ethanol. The column of adsorbent is extruded and streaked with alkaline permanganate (1 g. NaOH, 0.1 g. $KMnO_4$, 10 ml. water) using a brush, and making a light, uniform streak. The reagent turns brown where it crosses the zones, and then, more slowly, turns brown over the rest of the streak as it reacts with the residual ethanol. The zones are separated out and the streaked adsorbent shaved off and discarded. The upper zone contains the sorbitol. Each zone can be eluted with water (6 small portions, totaling *ca.* 15 ml.) and the carbohydrate present determined quantitatively if desired. In one experiment the sorbitol zone was eluted and the eluate titrated by periodate oxidation; *found*, 99.7% of the sorbitol. The lower, D-glucose zone by a reducing sugar determination showed 100% of the glucose.

Lew, Wolfrom, and Goepp (556) reported an experiment with lemon juice. The fleshy portion of a lemon was ground with acid-washed sand and centrifuged. One milliliter of the liquid was treated with 7 ml. absolute ethanol and the mixture centrifuged. One milliliter of the supernatant was chromatographed on a 0.9 x 6 cm. column of Florex xxx, using 2 ml

95% ethanol as developer. The column was extruded and examined by the brush method for zones. A streak made with 2,6-dichlorophenolindophenol reagent (5 mg. to 20 ml. water) showed a zone of ascorbic acid in the region 3.9 to 4.3 cm. from the top of the column. A streak with alkaline permanganate showed at least four zones. A streak with Congo red showed the presence of much acid above the ascorbic acid zone.

4. Elution Analysis

Three examples will be given briefly, the first (*A*) using a single eluent to remove one zone of material, and a displacer to sweep out all the remaining adsorbed material from the column; the second (*B*) using a "graded" series of eluents; and the third (*C*) an elution analysis employing a saturator.

A. Mair and Forziati (595,596) together with a group at the National Bureau of Standards have developed the use of silica gel for separating certain mixtures of hydrocarbons. Aromatic hydrocarbons are separately recovered from paraffins and naphthenes as follows. A column is prepared containing an amount of activated silica gel in excess of that which would adsorb all the aromatics present. The authors give methods for calculating this amount; a break-through curve is used to estimate the capacity of the adsorbent. For laboratory-scale work, 28- to 200-mesh silica is used, for large-scale experiments, 28- to 42-mesh. The ratio of length to diameter of the tubes used is generally not less than 30 to 1.

The mixture to be analyzed is allowed to pass into the column. As soon as the last has entered, it is followed by *n*-pentane, sufficient to remove all paraffins and naphthenes (which are not very strongly held). When all these saturated materials have been washed from the column the refractive index of the effluent reaches that of *n*-pentane. Methanol is then introduced. It displaces the aromatics, which are collected as a separate fraction. At the end of the displacement the refractive index of the effluent approaches that of methanol but may remain a little higher because the methanol tends also to displace a little water from the gel.

The paraffin-naphthene fraction is recovered by distilling off the *n*-pentane. The aromatic fraction is recovered by extracting it once with water to remove methanol, and distilling to remove *n*-pentane. In this step any residual methanol distills as an azeotrope with *n*-pentane. The adsorbent left in the column can be reactivated *in situ* by sweeping out the methanol with steam or an inert gas, and finally raising the temperature of the column to 180° to 200°C. while a stream of air or inert gas is passed through. A 52-ft. laboratory column for the separation of hydrocarbons has been described (596).

B. The use of a graded series of eluents can be illustrated by an experiment reported by Reichstein and von Euw (769). This is summarized in Table VIII-17. A crude acetylated benzene extract prepared in the process of working up a steroid mixture weighed 1.21 g. and was applied to a column containing 40 g. alumina. The column was treated with the solvents named in Table VIII-12, about 120 ml. of each being used. The percolates were evaporated down and the residues weighed. A further brief description of the fractions as reported by Reichstein and von Euw is given below.

Fractions 1 to 8 yielded a syrup from which, besides a little free sulfur, no crystals could be separated. Fractions 9 to 12 on crystallization from petroleum ether and recrystallization from ether gave 2.5 mg. of colorless leaflets, m.p. 184° to 185°C. These could not be identified. Fractions 13 and 14 were crystalline, m.p. 190° to 208°C.; recrystallization from acetone–ether gave 4 mg. of colorless, unidentified, irregular needles, m.p. 213° to 215°. Fractions 15 and 16 melted at 190° to 205°C. Recrystallization from ether yielded 6 mg. colorless prisms, m.p. 209° to 211°C., identified by mixed melting point as T-diacetate. Fractions 17 to 21 could not be crystallized. Fractions 22 to 26 gave small crystal masses, m.p. 185° to 200°C. Two recrystallizations brought the melting point to 205° to 206°C. Fractions 27 to 29 gave a mixture of needle masses (m.p. 190° to 210°C.) and leaflets (m.p. 201° to 213°C.) which could not be separated. Fractions 30 to 33 yielded on crystallization from acetone–ether *ca.* 40 mg. of M-acetate, m.p. 218° to 221°C. The fractions 34 to 38 consisted of mixtures which were rechromatographed and separated into U-diacetate and some Fa-diacetate. The last fractions 39 to 44 gave only brown syrup.

The rationale of this fractionation is apparent. The eluents are used in a graded series, starting with the weakest (this is why the other tables have been arranged, starting with the weakest eluent). Concentration gradation has been used to obtain small steps of elution increase between the functional gradations. If any question may be raised about the process it is that admixture usually increases eluent power, so that in admixture it may not be necessary to go to 100% of the second (admixed) liquid. This may not apply here. In any case, some recognition has been made of this in that the admixed liquid is added in initially small but gradually increasing ratio.

C. One of the effects that an eluent has, in causing a zone to move along a column (apart from the fact that it moves, and acts as a carrier, or solvent, for the adsorptive), is that it is itself adsorbed, and so competes with zone adsorptive for the available surfaces of the adsorbent. The competition is more effective with the less strongly held adsorptives—as indicated by the faster movement of these zones. This was pointed out by

TABLE VIII-17
Fractionation of an Extract (769)

Fraction No.	Solvent	Residue on evaporation
1	65% benzene + 35% petroleum ether	
2	80% " + 20% " "	
3	100% benzene	
4	" "	60 mg. syrup
5	" "	
6	" "	
7	99% benzene + 1% ether	
8	99% " + 1% "	
9	98% " + 2% "	23 mg. crude, from which 2.5 mg. leaflets, m.p. 184°–185°C.
10	97% " + 3% "	
11	96% " + 4% "	
12	94% " + 6% "	
13	92% " + 8% "	19 mg. crude, from which 4 mg. needles, 213°–215°C.
14	87% " + 13% "	
15	80% " + 20% "	44 mg. crude, from which 6 mg. T-diacetate, m.p. 209°–211°C.
16	70% " + 30% "	
17	55% " + 45% "	
18	40% " + 60% "	100 mg. syrup
19	100% ether	
20	" "	
21	" "	
22	" "	
23	99% ether + 1% acetone	23 mg. crude, from which 2 mg. crystals, m.p. 205°–206°C.
24	" " " "	
25	98% ether + 2% acetone	
26	" " " "	
27	97% " + 3% "	
28	96% " + 4% "	mixture
29	95% " + 5% "	
30	94% " + 6% "	
31	92% " + 8% "	40 mg. M-acetate
32	90% " + 10% "	
33	88% " + 12% "	
34	85% " + 15% "	
35	80% " + 20% "	
36	75% " + 25% "	mixture
37	70% " + 30% "	
38	60% " + 40% "	
39	50% ether + 50% acetone	
40	40% " + 60% "	

TABLE VIII-17 (*continued*)

Fraction No.	Solvent	Residue on evaporation
41	49.5% ether + 49.5% acetone + 0.5% chloroform + 0.5% methanol	
42	49% " +49% " +1% " +1% "	
43	48% " +48% " +2% " +2% "	
44	46% " +46% " +4% " +4% "	

Tiselius and Hahn (937), who suggested that if a column were pretreated with a certain concentration of such an eluent, and then used for chromatography, the movement of zones might be favorably influenced and additional control gained over the separation. The eluents used in this way are called saturators, and an advanced example of their use is taken from Porath (741).

The effect of the saturator appears to be to decrease the adsorption of adsorptive and straighten the isotherm (361,910). This kind of effect has been observed many times. As pointed out above, in its function the saturator may be indistinguishable from an eluent or eluting agent, or at times a displacer. In use, a saturator is chosen that is so firmly held to the adsorbent that it is not appreciably removed during the subsequent development, elution, or displacement steps. Enough of this is used to just saturate a desired portion of the more active sites on the adsorbent, leaving the less active sites for employment in the subsequent chromatography. A set of graded adsorbents can thus be prepared (exactly analogous to the gradations of alumina obtained by adding water, Section IV).

Porath prepared adsorbent in the following way. The charcoal, plus three times its weight of Celite, was suspended in a volume of boiling ethanol three times (in milliliters) the weight (in grams) of charcoal plus Celite. A weighed amount of stearic acid in a little less than the same volume of hot alcohol used above was slowly added to the suspension, which was then boiled down with continuous stirring to the original volume and allowed to cool. The mixture was filtered under suction and the adsorbent–Celite washed with three portions of 50% *n*-propyl alcohol, each portion having a volume twice (in milliliters) the weight (in grams) of the original adsorbent mixture. The adsorbent was then treated with a volume of distilled water at least ten times (in milliliters) the weight of the original mixture. The alcohol solutions were taken to dryness, and the unsorbed stearic acid was weighed; that taken up by the adsorbent was obtained by difference. It was found that 30% of the original weight of charcoal could be taken up as a maximum.

Six lots of charcoal were prepared containing 25%, 10%, 3%, 1%, 0.3%, and 0.1% stearic acid. These were packed in this order in sections

to form an adsorbent column, with the 25% charcoal as the top section and the 0.1% as the bottom. A concentrated pea-root exudate was applied to this column and developed with distilled water. According to theory, only the most strongly adsorbed material would be retained in the section saturated with 25% stearic acid, and the graded steps of the column would hold materials with decreasing adsorbabilities. After development the column was dismantled, and the sections eluted with 50% n-propyl alcohol. Paper chromatograms of the fractions so obtained indicated that peptides and aromatic ninhydrin-positive substances were held in the upper sections, amino acids in the lower and in the effluent. In the effluent most of the carbohydrates and all inorganic salts were found. Other separations were noted. The column could be regenerated by washing it with water. It may be noted that on a column so loaded with saturator, "partition" effects are to be expected (361).

5. Displacement and Carrier Displacement

A simple case of displacement was described as 4.A, above. In more complicated cases, usually applicable only to mixtures of homologs, or at least to groups of substances that all occupy the same kinds of sites on the adsorbent so that one may reversibly displace another, the mixture to be separated is applied as a mixed zone to a column of adsorbent. A displacing agent, more strongly adsorbed than any component of the mixture, is then applied in excess in solution. Acting as a sort of "piston" as it is passed into the column, it expels adsorptive from the surface, as described in Chapter IV. If all the components of the mixture compete for the same sites, then the most strongly adsorbed will gradually displace the next, and so on until the entire mixture is segregated into a set of zones, one in front of the other. This array moves down the tube (preceded by peaks of any fast-moving components that may be present) and so out into the effluent.

The great advantage of displacement analysis, where it works, is that it tends to reduce the tailing often found with ordinary elution analyses. (This is reduced when saturators are used.) But the difficulty remains of collecting zones cleanly when they emerge hard upon one another. Carrier displacement was designed to remove this difficulty. It is strongly analogous to carrier distillation methods and carrier crystallizations (22, 105,398).

In carrier displacement (carrier frontal analysis will not be discussed (936)) some inert material is introduced between each step in the displacement series so that zones of desired substances are separated from each other and can be easily collected. The carriers must be substances

easily separated from the desired zone materials. In practice it is difficult to set up a good carrier displacement (361), though Hagdahl and Holman (360) have made satisfactory separations of higher fatty acids using as carriers the methyl esters of these acids see Fig. VIII-10.

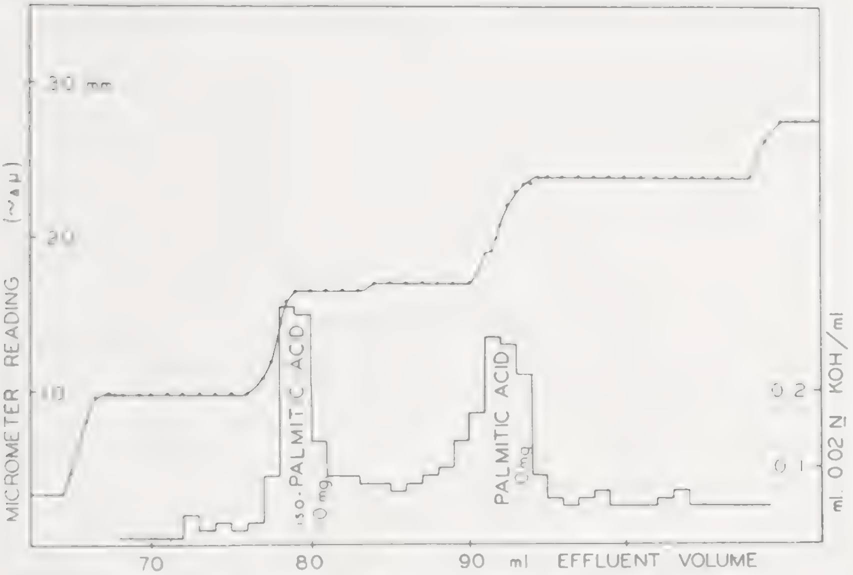


Fig. VIII-10. Carrier displacement analysis. The upper curve represents interferometer readings (see Fig. VIII-8) and is related to concentration of the effluent. The lower curve, obtained by titration, shows that the zones of acid occur as peaks within the fronts of the displacing carriers. There is some tailing, and overlapping of zones. (From R. T. Holman, private communication.)

Note, however, that the zones of the acids appear as peaks within the carriers, not as sharply marked off zones separated by pure carrier.

6. Gradient Elution Analysis

In an endeavor to decrease the tailing usually encountered in ordinary elution analysis, Tiselius and his co-workers (911) have introduced a technique whereby the effectiveness of the eluent is increased steadily instead of in steps (as in example 4.*B*, above). This was named gradient elution analysis (13) and shows evidence of being very useful not only in connection with adsorption but also with partition chromatography (12,13,148,607).

The idea behind this method is to maintain the rear of a zone in a stronger eluting medium than the front of the zone (933). The distribution of zone material between stationary and mobile phases is affected by the strength of the eluent. The eluent acts on the one hand to displace

the zone material from the adsorbent, and on the other to interact with it by dissolving it (see below). When the eluting power of the mobile phase is increased, the zone material is displaced toward the mobile phase. Thus in the gradient the rear boundary of a zone should be caused to move faster than the front, crowding up toward it, and producing a more compact zone.

The method is subject to the same limitation as the stepwise gradient system (example 4,*B*), in that oftentimes as a second solvent is added to a solution of an adsorptive (Section VII, Class 2 eluents) the eluting power goes through a maximum at some concentration as second component is further increased. Thus the admixture of second component in these cases should not go beyond the point of maximum eluting effect, and the gradient should be terminated at that point, or else the system of eluents should be changed.

The procedure is to place at the top of the adsorption column a reservoir of eluent which in general would be a dilute solution of a weak displacer in a solvent, or it might be pure solvent. As this flows into the column there is added to the reservoir, at a rate determined by the rate of flow of developer (eluent) out of the column, and usually equal to it, more of the displacer. Thus as dilute solution flows into the column, stronger solution is continuously produced above it. The reservoir is usually fitted with a stirrer, and the displacer is added continuously, or drop-wise, or in small portions, by hand or automatically (13,148,176, 239), and thus a concentration change is produced in the developer. It is rapid at first and slower as the concentration in the reservoir builds up (176). As Alm and co-workers point out (13), the gradient can be produced by change in concentration of a mild displacer, by change in pH, or by change in salt concentration. A gradient depending on temperature can be used for the same purpose, of course.

7. Radial Chromatography

This method was introduced by Brown (127) and has been used by many workers (205,622,623), employing as adsorbents powdered materials and impregnated papers.

The method in essence involves placing a layer of adsorbent between two sheets of glass, the upper provided with a central hole, or in a Petri dish, or in the depression of a hanging-drop microscope slide; or specially prepared on slides. The mixture is applied to this, and developed in the usual manner except that now the developer flows radially from the spot, yielding zones as shown in Fig. VIII-11. A thorough discussion of this method under the name of "surface chromatography" has been given

by Meinhard and Hall (622,623). These authors also describe convenient apparatus.

8. Retrospect

As has been pointed out by many authors and as must in general be obvious to any experienced worker, the optimum results using any of these methods require some knowledge of the system being used: that is, information about the relative distribution of the components of the mixture (whatever they may be) between different mobile and stationary

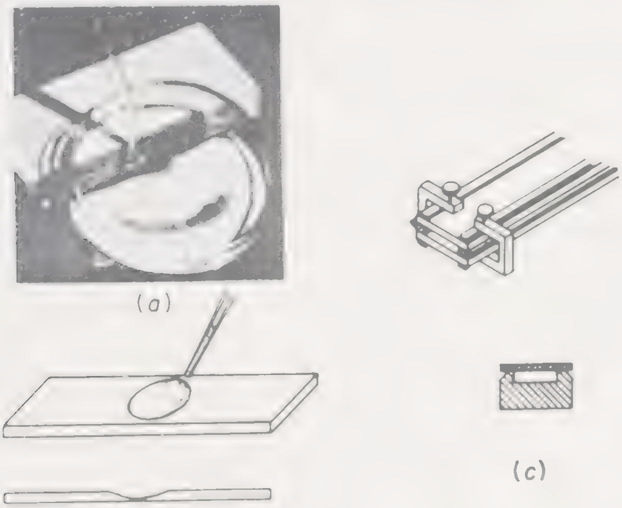


Fig. VIII-11. Apparatus for chromatography on a small scale, using strips or wedges of adsorbent. (a) A wedge of adsorbent is formed in a Petri dish; the substance to be separated is applied in the center of the straight edge (205); (b) the adsorbent is placed on the ground depression in a "hanging-drop" microscope slide; (c) the column is formed between two glass plates separated by spacers, or in a covered trough (60).

phases. Seldom does the first attempt at a separation give optimum results (a condition that wouldn't be recognizable with one experiment, anyway). What may happen, with easy separations, is that a sufficiently good separation is obtained, and thus for practical reasons, no further attempt may be made to determine optimum conditions. However, in the field of adsorption it is not always easy to reproduce the results of other laboratories, so that the worker may need to determine his own conditions to some extent even in these circumstances.

It is because of these considerations that a Chapter XIV has been written to show some of the ways of attacking problems. The complexity of these phenomena, as reflected in the principles stated in the next

section, must be welcomed as necessary adjuncts to the power of chromatography as a separation method.

IX. FACTORS THAT AFFECT R VALUES AND THE SEPARATION OF ZONES

1. General

Practical considerations prevent devoting the space to adsorption phenomena given in a previous volume (169). For this reason, this section is highly condensed and statements are made, and rules suggested, without detailed documentation except references to the literature. Throughout this section the two cardinal principles of practical chromatography must be born in mind: *(1) separations rest on competitions between all the components of the system; (2) all rules for choices of stationary and mobile phases, except those based on thermodynamic considerations, are contingent.*

Consider the simple case of a stationary phase (an adsorbent surface) (**S**), a mobile phase (**M**), and two components (adsorptives) C_i and C_j. Interactions will occur between all of them: Table VIII-18, a total of

TABLE VIII-18
Interactions in a Simple Case of a Binary Mixture, a Developer, and an Adsorbent

For this, \ these compete	S	M	C_i	C_j
S		X	X	X
M	X		X	X
C_i	X	X		X
C_j	X	X	X	

The stationary phase is S, mobile **M**, and components of the mixture C_i and C_j. Reading across on any line, such as the first, "for the stationary phase, molecules of mobile phase and components *i* and *j* are in competition," or on the second line "for molecules of mobile phase, molecules of stationary phase and of components C_i and C_j compete."

Note. Not explicitly shown are those *associative* interactions in which **M**, C_i, C_j molecules interact with their own kind.

six reversible pairs. Some are of temporary importance, in that as soon as the zone C_i draws away from the zone of C_j, one pair of interactions disappears. In more complicated cases there are correspondingly greater numbers of possible competitions (owing to interactions). It may be noted

that interactions of the C_1:C_1 type are not in general negligible. The existence of constant adsorption mixtures (adsorbotropes), among other phenomena, warns against neglecting such interactions (39,406,697,998). It is because of the multiplicity of possible interactions, combined with the paucity of data about how factors such as temperature, geometry of the interacting molecules, actual state of the interacting molecules (e.g., whether solvated, associated, dissociated) affect each of the tabulated interactions, that we must recognize the contingency of most of our rules and generalizations.

The thermodynamically based generalizations are, of course, sound, but in general (except in the developing field of gas chromatographies) they are not very useful because of lack of data—especially when mixtures of unknown substances are faced by the chromatographer.

2. Analysis of Factors That Affect R Values, and Separation of Zones

In Table VIII-19 is given an analysis of factors that may affect **R** values. The velocity of movement of a zone relative to that of the developer (Chapter IV), **R**, was related by LeRosen (542) to the time spent on the average by a molecule in the mobile phase between adsorptions (T_M) and in the adsorbed state (T_S) through the ratio

$$\mathbf{R} = T_M/(T_S + T_M)$$

This relationship serves conveniently for the analysis of the factors that affect **R**. The analysis follows the analogous scheme in Chapter VII, and the two are obviously so closely related that except as we deal here with a solid surface (rather than a sorbed film) as the stationary phase, a good deal of the discussion from Chapter VII is applicable here.

A. BULK FACTORS

1. The bulk factors related to the column itself, the uniformity of packing, diameter: length ratio, and velocity of flow of mobile phase have been discussed chiefly in Section IV. They affect the separation by controlling the sharpness of the fronts and the disposition of the rear boundaries of zones.

2. The bulk factors related to the particles themselves, the effects of size and shape and porosity on packing of the column and on the definition of the zones has been discussed in Section IV. The purity in terms of homogeneity of adsorption sites (really, narrowness of range of different kinds of adsorption sites, for only in a liquid–liquid interface may true homogeneity be expected) will be discussed in Section B(*1*) below. It is a very important factor, because it spells the difference between

TABLE VIII-19

Factors That May Affect **R** Values and Separation of Zones

A. Bulk factors (operate chiefly through S and **M**)		B. Molecular factors (operate through S, **M**, and C_i)	
Of column	*Of adsorbent particles*	*Factors that influence T_S*	*Factors that influence T_M*
Uniformity of packing Diameter vs. length Velocity of flow of **M**	Size, shape, porosity, purity	(operate from the side of the adsorbent) C_i:S interactions, **M**:S interactions	(operate from the side of the solution) **M**:S interactions C:**M** interactions $C_i:C_j$ interaction

Note that in general the factors that increase T_S will decrease T_M, and vice versa. Here S refers to stationary phase; **M** to mobile phase; C_i, C_j to any two components of the mixture to be separated, e.g., to two adsorptives. The average times spent by a molecule of an adsorptive in the stationary phase, and in the mobile phase between adsorptions, are given by T_S and T_M, respectively. The interactions are classified in Table VIII-18.

Langmuir-type isotherms (homogeneous sites), which are desirable to have but rarely found in adsorption chromatography of solutions (169), and Freundlich-type isotherms (wide range of differences in kinds of sites), which are the kind normally encountered in this kind of chromatography (169,1004).

B. MOLECULAR FACTORS

Factors that increase T_S cause the molecules of adsorptive to be more strongly adsorbed. At the same time they decrease T_M, so that all these factors could be discussed in terms of T_S. However, it is convenient to separate them into factors that operate predominantly from the side of the adsorbent surface (*1*) and into factors that operate from the side of the solution (*2*).

(1) Surface of the Adsorbent. The surface of the adsorbent being limited, sites occupied by mobile phase molecules cannot in general (unless a second layer is formed, which undoubtedly does occur in some cases) also be occupied by adsorptive, but they may be accessible through (competitive) displacement to the adsorptive. It must be noted that there is often some freedom of movement of adsorbed molecules about a surface, from site to site, as well as desorption under the impacts of ambient molecules (72,276,403,879). Therefore insofar as mobile phase molecules compete effectively for the stationary phase with molecules of the adsorptive they will decrease T_S and increase **R**. This is one half of the picture that correlates solvents, developers, eluents, saturators, and displacers

The other half is the $\mathbf{M}:C_2$ interaction, so the entire picture will be presented in the next part (*2*).

Since the surface of the adsorbent interacts with molecules irrespective of their labels as $\mathbf{M}$ or C_1 or C_2, we can drop these designations and discuss the factors that bring about adsorption.

Two classes of these have been discussed broadly in Chapter III: the physical and chemical interactions. They are not further treated in detail here, but an attempt is made to state helpful correlations.

Adsorbability—tendency to be adsorbed—may be related to *chemical structure* in a general way. Within a given homologous series there is usually an order of relative extent of adsorption (from a given solvent to a given surface) which supports the concept of homology. That is, usually there is no haphazard arrangement of the substances when the relative extents of adsorption are compared, but adsorption either increases or decreases in a regular manner with increasing number of CH_2 groups. If a very irregular arrangement were to be observed it would throw some question on the techniques employed in the experiment and cry for verification.

Whether the observed order follows the Traube rule, adsorption increasing regularly and strongly with number of carbon atoms, or is the reverse of the Traube rule, or is not variable with number of carbon atoms, seems not to be solely a function of the chemical structure of the adsorptive but rather to depend upon the adsorptive-surface interactions and to be influenced by the solvent. Thus it may be recalled (160) that from petroleum ether the order of adsorption on a charcoal is lauric < myristic < palmitic < stearic acids; on an alumina, lauric = myristic = palmitic; and on a silica gel stearic < lauric acid.

The observed extents of adsorption between substances belonging to different homologous series are of course functions of the chemical structures as long as a given solvent and adsorbent are compared. This is shown in the extensive study by Linner and Gortner (560) in which fatty and substituted acids were adsorbed from water onto Norit charcoal. The authors concluded that under these conditions the introduction of OH into an organic acid decreased its adsorption to an extent depending upon the acid, and NH_2 produced a still greater effect. This is understandable on the basis that OH will increase the interaction with the water. Also, when substituted in the *alpha* position, OH increases acid dissociation. Either of these effects will lessen the tendency toward escape from the solution to the surface. The NH_2 being still more basic than the OH forms a cation NH_3^+ even in water solution, and the zwitterion formed with the carbonyl shows great solubility in most cases compared with the unsubstituted acid. A second OH group further decreases adsorption,

from water, but not to the same extent that the introduction of the first OH did. Thus the observed extents of adsorption are glycolic < lactic < propionic, and tartaric < malic < succinic. The introduction of a keto group tends to decrease the relative adsorption; the orders found for extent of adsorption are pyruvic < propionic; levulinic (γ-ketovaleric) < valeric acid. That glyoxylic acid is more strongly adsorbed than acetic does not seem to fit here; it may indicate that some depolarizing effect occurs in glyoxylic acid. The keto groups would be expected to interact through hydrogen bonding with the water and so lessen the tendency of the adsorptive to escape from the solution. In each case the position of the substituent relative to the carboxylic group must also influence the extent of the adsorption.

Introduction of a double bond was shown to increase adsorption in some cases. Orders of adsorption found were methylsuccinic < itaconic < citraconic < mesaconic acids; maleic < succinic < fumaric acids. Introduction of halogen should have an effect similar to, but less marked than, introduction of OH. This is shown in the order tartaric < malic < dibromosuccinic (nearly equal to monobromosuccinic) < succinic acid. In general it might be expected, then, that for substituted aliphatic acids the adsorption from water to charcoal would be, in terms of the substituent groups, $F < Cl < Br < I < OCH_3 < C_6H_5 < H < CH_3$, other things being equal, for acids with substituents on the same carbon atom. This is the order of decreasing "negativity" of the groups as judged by the effect on the dissociation constant of the acid. However, this does not mean that the effect of the substituent is solely related to its effect on the dissociation of the carboxyl group, since geometric and other effects must also influence the adsorption.

A second generalization may be made that, other things being equal, introduction of a negative group should increase the extent of adsorption of the substance from a nonpolar solvent. The data of Heymann and Boye (396) generally bear this out.

There appears to be no good evidence to show that a quantitative relationship can be deduced at present between extent of adsorption at the solid–solution interface and molecular structure. Evidence is good, however, that certain limited, contingent, qualitative statements such as those above can be made (see Chapter XIII). At the solution–vapor interface, Traube's rule correlates adsorption with structure *within* the homologous series.

The *shapes* of molecules should affect extent of adsorption especially where porous adsorbents are involved. However, it is difficult in many cases to differentiate adsorption effects ascribable to molecular shapes from those due to *size* (or *volume*). One might think of shape factors as

applying to the comparison of the properties of a linear molecule such as 1-hexanol with the more compact cyclohexanol. However, Langmuir (522) showed that the hydrocarbon chain portion of such a "linear" molecule as hexanol must be to some extent curled up when the molecule is in solution so as to offer to the solvent the minimum surface area. Treloar (944) has concluded that in its most probable shape a hydrocarbon molecule is spherical (917), and Ward (969) has extended these ideas to the shapes of small polar molecules (of fatty acids) in surfaces of their solutions. In such cases, therefore, differences in adsorbability may not readily be ascribable to shape if both molecules take the form of a somewhat flattened sphere in the surface. However, as Ward (969) has pointed out, although in solution surrounded by like molecules, the shape is determined solely by configurational probability, the most probable shape approximating a spheroid; in the surface both entropy and surface energy factors influence the shape. At an immobile interface topological factors may be very important also. In this connection it may be recalled that although *n*-hexane is more strongly adsorbed on silica gel than is cyclohexane, the difference in extent of adsorption between the two is small (596). When branched-chain molecules are compared with each other and with *normal* molecules, then some of the differences observed may be ascribable to shape. Thus, Weber and Sternglanz (970) found that among lower aliphatic alcohols those with normal chains are more capillary-active in the surface of their water solutions than those with branched chains. Of isomers with branched chains, those with the branching farthest from the polar OH group are more surface-active. Unsymmetrical isomers are more active than symmetrical isomers, and the surface activity increases from tertiary to secondary to primary compounds.

In cases of adsorption from solution to solid surfaces where different classes of molecules are compared, differences in extent of adsorption which are laid to differences in shape (261) need to be scrutinized. Too little is known about the shapes of molecules. In those cases, however, where molecules are constrained to a surface of a homogeneous substrate, and are oriented in a deducible manner, good evidence has been obtained that shape may play an important role in relative adsorption. The subject is discussed by Adam (7) in connection with films of insoluble substances on the surfaces of liquids.

It is understandable that molecular volume may influence adsorption in important ways. Thus it might be expected that in mixtures of carbon tetrachloride and benzene (for example), and because their molecular volumes are nearly the same, the adsorption curve over the entire concentration range should be symmetrical. Yet the adsorption of carbon

tetrachloride is much less than that of benzene—an effect possibly ascribable to the polarizability of the benzene (396). Ilin and Kiselev (431) found, in studies of the heat of wetting of steam-activated, sieved, and dried wood charcoal, that the time for complete wetting increased with the molecular volume of the adsorptive. Baum and Broda (46) found that solutions of acetylated celluloses and sugars did not reach equilibrium with alumina and charcoal even in several months contact. This was presumably due to the large molecular size of the adsorptives. Bruns (131) concluded that the reversal of Traube's rule, sometimes found for adsorptions on charcoal, was caused by the presence of pores which were not accessible to compounds with large molecular volumes. (See also (515,684).)

That there should be a relation between molecular volume and extent of adsorption would follow on two lines of reasoning. Several workers (46,120) have pointed out that adsorbability should tend to increase with chain length because of the increase of cohesive forces up the homologous series (for example, as manifested in the regular increase in heat of melting). The apparent molecular volume of a solute in solution should also be correlatable with extent of adsorption through the evidence it gives of the interaction between solute and solvent (44).

Hydrogen bonding of the adsorptive with the adsorbent would tend to increase the extent of adsorption. The subject has been extensively investigated by Hoyer (426–428) and Kipling and his co-workers (77,78).

(2) Molecular Factors That Operate from the Side of the Mobile Phase. Factors that affect the **M**:**S** interaction can be derived from the discussion above. Adsorption is decreased, and $T_{\mathbf{M}}$ is increased, when the mobile phase interacts more strongly with the adsorbent.

$T_{\mathbf{M}}$ is also increased by interactions of the **M**:C_1 type, which solubilize the adsorptive in the mobile phase. This is the other side of the picture, referred to above, that connects all the different functions of mobile phases. An analysis of mobile phases is given in Table VIII-20. This brings together in a functional way the types of mobile phases. The observations of Williams, Hagdahl, and Tiselius (1004) about saturators and eluents fall into place as the two competing effects on a solute from the adsorbent and the mobile phases. Thus, as they point out, changes in the Langmuir constant k_1 (equation (6b) in Chapter IV) parallel changes produced by eluting agents. This constant can be related to the affinity of the adsorbent for the substance, and we see here the *solubilizing effect* of the eluent (*relative* to its displacing effect!). Changes in the k_2 constant parallel, they found, the effects produced by a saturator. The constant k_2 is related to the number of available sites for adsorption, and the saturator, more

TABLE VIII-20
Mobile Phases

- *Gases or vapors*
  Carriers in gas chromatographies; may function as shown under liquids, other category; see Chapter V.
- *Liquids (solutions)*
  Carriers in adsorption and liquid–liquid distribution chromatographies
  - Function
    - [a] Solvents
    - Developers
    - Eluents
    - Saturators
    - Displacers

Order of increasing interaction with adsorbent →

[a] Note that the position along this line is not meant to have any quantitative implications.

strongly adsorbed than the eluent (and present in smaller amount, usually) acts to block some of them, so that fewer are available to the adsorptive. The authors sum up the relation with the proposition that "saturators and ideal displacers act by blocking adsorbent sites but that eluting agents chiefly act by reducing their affinity without blocking them" (1004). In other words, one exerts its effect chiefly on the stationary phase; the other in the mobile phase.

These authors have brought the understanding of behavior described by the Freundlich isotherm to a focus by using Hinchelwood's expression of the Freundlich isotherm as a sum of Langmuir terms:

$$q = \Sigma\, k_1 k_2 c/(1 + k_1 c)$$

Here again, the effect of eluent is to increase the amount of solute (adsorptive) in the mobile phase, relative to that in the stationary; whereas the effect of a saturator is to block the less wanted sites on the quite inhomogeneous surface. These sites may include pores from which adsorbed solute is not easily recovered (one possible cause of tailing). The authors also point out that the effect of the eluent may not reduce very much either the capacity of the adsorbent or its selectivity.

Certain considerations may be presented that will help to estimate where in the above gamut from solvent to displacer a substance will fall, and since its behavior in solution will be independent of whether it is labeled adsorptive or mobile phase (the difference here being a matter mostly of amount and concentration), we will drop the distinction for the rest of this section whenever it is convenient to do so, remembering that both compete for the surface and both interact with each other, and both may associate (an additional interaction not shown explicitly in Table VIII-18).

Association of the molecules of the adsorptive has been thought to affect the extent of an adsorption. Thus Bakr and McBain (26), in studying the adsorption of toluene and acetic acid on charcoal from sugar and on extracted animal charcoal, found acetic acid less adsorbed at high temperatures than toluene. At 245° C. practically no acetic acid, but considerable toluene, was adsorbed. This was related by these authors to the known effect of high temperature in dissociating the acetic acid dimers. Heymann and Boye (395) suggested that the adsorption of benzoic acid from nitrobenzene onto charcoal is greater than that from acetone or nitromethane because the benzoic acid is exceedingly strongly associated in the nitrobenzene.

Certain associations of solute and solvents to form complexes with special adsorption characteristics have been adduced by Doss and B. S. Rao (241), K. S. and B. S. Rao (757), and N. S. Rao and S. Jatkar (759) to explain the shapes of isotherms of adsorption from binary mixtures over the entire concentration range.

It is enlightening to recall that when a polar substance is dissolved in a nonpolar, as acetic acid in toluene, the polar tends to associate (in this case through hydrogen bonding, to produce chelated dimers, and possibly trimers) becoming to some extent *depolarized*, and thus less polar and more like the solvent. In the above cases, the charcoals preferentially adsorb nonpolar molecules.

Hydrogen bonding may enter the picture of mobile phase interactions. As just pointed out, hydrogen bonding between solute molecules usually results in a depolarization of the molecule, which may or may not militate against adsorption. In the case of gases this associating effect would lead to increased condensability, which would tend to increase relative adsorbability. Depolarization of a substance through chelation would increase its solubility in a nonpolar solvent. Bonding of the solute with the solvent may be responsible for abnormally large solubility, with concomitantly lower tendency to escape from the solution into an interface.

The degree of polarity of a molecule can be judged from the nature of the groups present in it, or of the bonds present, or from the *solubility behavior* of the substance. On this latter basis a substance is called hydrophilic if it is fairly water-soluble. This usually implies a somewhat polar character to the molecule, or the presence of rather polar groups or bonds, or the absence of relatively large nonpolar groups. *Hydrophobic* substances are not appreciably water-soluble, and likely to be nonpolar. To look at these properties from another point of view, hydrophilic substances are sometimes spoken of as *lipophobic* or *oleophobic* (oil-insoluble), and hydrophobic as being *lipophilic* or *oleophilic*. The general terms are *lyophilic* and *lyophobic*.

The term hydrophilic, besides implying water solubility, also may imply water wettability. In any case it implies a polar nature to the substance. If a substance is wetted by water, or swells in water (indicating penetration of its structure by the water molecules), or dissolves in water, then it has some polar character, but absence of these behaviors may not necessarily mean absence of polar character *in the molecules* of the substance. Thus a surface largely composed of CH_3 groups, as in an oriented fatty acid film, may be quite hydrophobic, the hydrophilic end of the molecules being hidden.

In view of these relationships it would be expected that *hydrophilic substances would be less well adsorbed from solution in each other than in hydrophobic substances.* An adsorbent that is readily wetted by a substance interacts to some degree with that substance and must adsorb it to some extent.

3. General Summary

Relative polarity was discussed in Chapter III. In general, polar character in a molecule implies an ability to interact with other molecules (of the same or different kind). The interactions are not specified. They include dispersion, orientation, induction, ion-dipole, hydrogen bonding, and other "physical" interactions and also even chemical interactions. The polar character increases with the intensity of the interaction; with the extent of departure, one might say, from "ideal" behavior. Since there is no easy way of giving a quantitative meaning to this term, the polar character of one molecular species is judged *relative* to that of another. This can be done on a "more or less" basis with the use of chemical principles such as were discussed in Chapter III.

On considering adsorbents, solvents, and adsorptives some useful rules may be derived, for not only are such important properties as *relative* solubility and *relative* wetting power predictable from *relative* polarity, but also the tables of elution series show some order, their contingent nature becomes more apparent, and they become more useful. It would appear, for example, that *generally the order of increasing extent of adsorption on silica is the reverse of that on sugar charcoal.* The more polar the molecule the more strongly is it adsorbed on polar silica, and the less polar, the more strongly adsorbed on nonpolar charcoal. A further generalization which holds fairly well is that other things being equal the order of adsorption on silica will hold for other polar solids, such as alumina, silicic acid, titanium oxide, calcium carbonate; and that the order of extent of adsorption to charcoal will hold also for other nonpolar (in adsorption behavior) solids, such as talc, stibnite, graphite, and paraffin-like solids (e.g., some "plastics").

But in view of the definition of relative polar character it is not surprising to find variations from the rules. Some charcoals can show exceedingly polar surfaces; molecules like benzene are more polarizable than others; acetone and similar carbonyl compounds show marked acceptor power in hydrogen bonding, while chloroform and carboxylic acids show donor ability. In a particular case, one of these properties may have an exaggerated effect on the adsorption, more than would be predicted from the rote application of rules. Thus the tables and rules guide without dictating. One balances the particular case against the general rule.

After balancing the relative polar characters of adsorbent, solvent, and adsorptive, the recognition of the competition for the limited surface of the adsorbent completes the list of the more important factors that enter the prediction. Again the emphasis is on *relative* behavior. There is only a limited area available to the solvent and adsorptive molecules. Geometrically speaking, it may be available equally to both molecules, but of course spatial factors may make more surfaces available to one than to another molecule. Barring extreme cases the distribution between bulk phase and interface will be governed by the relative polar characters of solvent and adsorptive vis à vis adsorbent.

4. General Rules for Predicting Relative Extent of Adsorption

With these considerations in mind, some useful rules may be presented to guide the prediction of relative extents of adsorption.

Rule 1. Greater adsorption to a surface will occur from that system which produces the greater lowering of interfacial tension. This rule follows from the Gibbs relation. The interfacial tension is a manifestation (in part) of the unlikeness of the two phases. The adsorptive, as the result of adsorption, tends to reduce this unlikeness and to make the transition in properties at the interface less abrupt. A corollary to this rule is that *a liquid which is highly adsorbed by a solid will show a low interfacial tension against the solid* (and a high adhesion tension, or spreading pressure).

Rule 2. The extent of adsorption is usually greater from solvents in which the adsorptive is less soluble. Lundelius (572) attempted to state this rule quantitatively. The phenomenon has been given explicit chromatographic application by Tiselius (932) and by Holman and Hagdahl (416,417). A corollary to this rule is that *for a given adsorptive the better solvent will make the better eluent.* This rule can be expected to hold well, particularly in those cases in which the solvents being compared show about the same interfacial tension toward the adsorbent being used (36). The various rules by means of which solubility may be pre-

dicted and the tests for determination of solubility are useful in conjunction with this rule.

Rule 3. The more polar the substance the more extensive its adsorption at a polar surface, other things being equal. A corollary to this rule is that *a polar substance will be better adsorbed from a nonpolar solvent than from a polar one.*

Rule 4. The less polar a substance the more extensive its adsorption at a nonpolar surface, other things being equal. But the range of values observed is usually not so wide as that found with polar solids. This may be one reason why polar solids are more favored for adsorption separations (except special separations such as decolorization); they are available with a wider range of activity.

Rule 5. Extent of adsorption changes in a regular manner along an homologous series. This rule is implicit in the concept of homology. Taken with Rules 3 and 4 a corollary follows: *since relative polarity decreases up an homologous series, extent of adsorption to nonpolar surfaces should increase up the series.* This is a restatement of the Traube relation. Another corollary might be stated: *since relative polarity decreases up the homologous series, extent of adsorption to polar surfaces should increase down the series.* This is a restatement of the Holmes reversal of the Traube rule. Rule 2 would warn, however, that, since solubility usually decreases (with any solvent) with sufficiently great increase in molecular weight, this second corollary must be applied with discrimination.

X. USE IN TESTING PURITY

The essential parts of this section are transferable to other kinds of chromatography.

By "test of purity," we really mean "detection of impurity" (165), for there is no sure chemical way of proving a substance pure (926). We can show with any confidence only that if the substance contains impurities of this or that kind, they cannot exceed thus and such an amount. If crude methods are used to find impurities, then the degree of purity can be stated only to a crude approximation. With progressively more acute methods, we become correspondingly more able to detect impurity. The definition of purity is based on the operations used to detect impurity, and we define a degree of freedom from impurity disclosed by the measurements. Purity is thus a relative matter, and any statement that purports to define absolute chemical purity is operationally meaningless.

The most subtle and reliable tests of purity depend on two operations: *attempts to separate the substance into fractions that are different,* and

tests of identity applied to the fractions. The melting point, for example, and a mixed melting point, are tests of identity of the substance with another that has been reported. Some qualitative measure of purity may be obtained from the melting range. But a more profond test that involves the melting point and fits the criterion for a test of purity is the cooling curve (859). If a substance is pure it will pass through the fractional solidification (crystallization) involved in taking a cooling curve in such a way that, barring supercooling, the temperature at which crystallization first starts will be the same as that at which the last material solidifies. The cooling curve method combines an attempt at separation (fractional crystallization) with a test of identity of the putatively separated fractions (freezing temperature). It thus meets the requirements laid down for the most acute type of test of purity. Such a test of purity is sufficient to itself, as far as it goes, in that it does not require a standard, or reference sample of the substance, as does an ordinary melting point. Swietoslawsky ebulliometry (902) and the Craig countercurrent distribution (198) (CCD) test are analogous in their spheres to the cooling curve.

These tests are, however, subject to the limitations that constant-distribution mixtures impose. Thus, a eutectic mixture behaves like a pure substance in the cooling-curve apparatus; and an azeotrope like a pure substance in the ebulliometer. Analogous limitations beset other distribution methods. No single test of purity, even though it meets the requirements laid down, is conclusive. The weight of evidence becomes greater when several tests are applied and met. Thus, in ebulliometry, measurements at more than one pressure are needed to exclude azeotropes; with solvent distributions, use of several different solvent pairs.

Chromatography obviously meets the requirements for the most acute type of test of purity. Like a cooling curve or an ebulliometric measurement, it combines an attempt at a separation (differential countercurrent adsorption) with a test of identity (the appearance of zones with different R values). However, the existence of constant adsorption mixtures warns that, as with the other methods, a more conclusive test requires the use of a different adsorbent and solvent system—preferably a system quite different in polar nature from the first.

Cases are known, however, where two different substances may behave the same way, and a single pure substance may give two or more zones on a column. This latter may occur when development conditions are changed during a chromatography or when other forces intervene. The occurrence is, however, rare, and is sometimes readily recognized, as is described below. Analogous difficulties are found with other methods of testing purity.

A few examples will serve to indicate the acuity of chromatographic methods in separating out small amounts of one substance from large amount of another. Martin and his co-workers (193) examined an authentic sample of Thudichum's "glycoleucine" from spinal cord and found it to be leucine. They also showed that not more than 0.03% of the nitrogen of hydrolyzed spinal cord could be present as *nor*leucine since when 0.5 μg. of *norleucine* was added to 1.2 mg. of hydrolyzate it could be clearly recognized with paper chromatography, but none could be detected in the spinal cord hydrolyzate alone. Wolfrom and his co-workers (556) showed that 2% of D-mannitol can be separated when admixed with sorbitol. The adsorbent was Florex xxx.

Among lipoid-soluble substances, Winterstein and Stein (1018) were able to detect and separate 2 mg. of dipalmityl ketone in 300 of hentriacontane which showed the theoretical analysis and a good melting point. They concentrated 0.5% ergosterol in admixture with cholesterol by chromatography on alumina. In this laboratory also (1017) 0.05% carbazole was isolated from anthracene. The purified anthracene showed its blue fluorescence, which is known to be quenched by 1/30,000% of naphthacene. This latter substance had been present in the original sample of anthracene to the extent of 0.5%. Mair and his co-workers (594–6) have shown that the aromatic hydrocarbon content of a mixture of paraffins, naphthenes, and aromatics can be determined to within ±0.2%. Zechmeister and McNeely (1037) were able to determine 1 to 2% of *cis*- or *trans*-stilbene in admixture with the other form. Cassidy (172) was able to show the presence of 0.67% ester in a sample of mixed fatty acids, by chromatography from petroleum ether on Darco G-60 charcoal. Claesson (178) could demonstrate 3.9% lauric acid and admixture with myristic.

An interesting example of precise work with natural mixtures has come from the laboratory of Ruzicka (747). From 82.2 g. of non-saponifiable material, representing 1500 kg. of pig spleen, these workers isolated 25.4 mg. of the triterpene friedelin. This represented 0.03% of the non-saponifiable fraction and was shown to be an impurity from cork stoppers into contact with which the material had come.

A case of two substances that behaved the same chromatographically may be found in an observation of Bauernfeind and co-workers (45). They found two pigments which were chromatographically identical by mixed adsorption on alumina but which were not identical spectrophotometrically. Sometimes a pure substance may give several zones in a chromatogram because it is altered during the process (1031). A pure *cis* compound may be altered to a mixture of *cis* and *trans*, and yield two zones. The classical observation of this effect was that carotenes could be isomerized by chromatographic adsorption (316). This phenomenon

exploited in the hands of Zechmeister and his students (1029,1032), led to their important work on the configuration of the polyene pigments.

XI. LARGE-SCALE CHROMATOGRAPHY

This application has received considerable attention in various industries faced with difficult separation problems (815). In the processes known as Hypersorption (63,464) and Rotosorption (791a) mixtures have been separated on a large scale. For example, small amounts of ethylene in a hydrogen–methane stream may be recovered. In one case the feed gas contained 4.5 to 6.0 volume % ethylene. The product ethylene showed 92 to 93% purity, with 0.1% maximum methane contamination. The hydrogen–methane stream contained less than 0.1% ethylene. One of these units processed 1,800,000 std. cu. ft. feed gas at 75 lb./sq. in. gage per day. In this tower, the gas passes upward through a bed of charcoal which is in motion downward. The motion, which may be as high as 32,000 lb./hr., is ingeniously controlled so that spent charcoal, removed at the foot of the tower, can be stripped of adsorptive, reactivated, and returned to the top of the tower. These data are from a Hypersorption installation.

The chromatographic method has been used in work on vitamins (1000), plant pigments (846,1000), antibiotics (718), and many other substances. Industrial applications will not be taken up here. Three very thorough reviews are available (972,975,1008).

Ion Exchange

I. PRINCIPLE

The stationary phase comprises a polymeric structure with fixed charge sites in the neighborhood of which oppositely charged ions reside. These latter may be exchanged with similarly charged ions in the mobile phase, electroneutrality being preserved. The exchanger may also operate to sorb neutral material, and it may produce chemical effects, both properties leading to the corresponding types of chromatographic behavior. The essential feature of ion exchangers is that one ionic species, cation or anion, is *fixed* and yet *accessible* to ions in the ambient medium (350).

II. CLASSIFICATION

Ion exchangers are so varied in their natures and properties that any thoroughgoing classification at all levels of behavior would be extremely complicated. A simple scheme for organizing the contents of this chapter is shown in Table IX-1.

Very little will be said about the inorganic exchangers, clays and zeolites (natural and synthetic). They are of tremendous practical importance, but the field is so complicated that specialized books must be consulted regarding them (14,28,676).

The organic exchangers, anion and cation, are mostly commercial products, many of which are being improved, others of which are being discontinued, and new representatives of which are continually becoming available. It is in some cases difficult to obtain information about these resins. The field is in a state of flux. It is necessary, therefore, to refer to reviews (695,768,829), symposia (432), and discussions, etc. (175,511a, 676,695a,802), for the latest information—some of which will undoubtedly be of passing value—and to try in this chapter to deal mostly with principles.

The ion exchanger sites are of two kinds, anion and cation. These may be combined in the same exchanger, or the two kinds of exchangers may be combined in a mixed bed. Each of these kinds of exchangers may act essentially as exchangers, as catalysts, or as sorbents; or any two or all three properties may be displayed simultaneously.

The most practical further way to class the exchangers may be in terms of dissociation behavior of the acid or base forms. Thus cation

TABLE IX-1
Classification Scheme for Ion Exchangers

- Inorganic backbone
  - (Classification here analogous to that under *Organic*, but properties and applications limited)
- Organic backbone
  - Cation exchangers
  - Anion exchangers
    - Principal behavior as
      - Exchanger
        - Cation: Strong acid, Weak acid, (chelater)
        - Anion: Strong base, Weak base, (chelater)
      - Catalyst (Sections V, X)
      - Sorbent (Sections VI, VII X)

exchangers may be classed as strong acid or weak acid types; and the anion exchangers as strong base and weak base. A further function of many specifically designed exchangers is a chelating function superimposed on the exchange function. Very likely the most fundamental classification of ion exchangers is based on the position of the key element of the functional group in the Periodic Classification, as shown in Table IX-2. By key element is meant the element other than O or H that attaches to the polymeric backbone of the exchanger.

As stated above, two types of exchange behavior are shown by ion exchangers: cation exchange and anion exchange. Both inorganic and organic exchangers of each type are available. The behavior of cation exchangers may be generalized:

$$R—A_1^-M_1^+ + M_2^+ + A_2^- \rightleftharpoons R—A_1^-M_2^+ + M_1^+ + A_2^-$$

where R is a polymeric structure to which is chemically bound (covalently) the anionic group A_1^-; M_1^+ and M_2^+ are cations; A_2^- is an anion which may or may not be functionally the same as A_1^-; and the interactions between A^- and M^+ are essentially ionic in nature. The anion exchangers behave, in general, thus:

$$R—B_1^+A_1^- + B_2^+ + A_2^- \rightleftharpoons R—B_1^+A_2^- + B_2^+ + A_1^-$$

or:

$$R—B_3: + B_4^+ + A^- \rightleftharpoons R—B_3:B_4^+A^-$$

Here R is a polymeric structure to which the cationic group-B_1^+ is covalently attached, while A_1^-, A_2^-, and B_2^+ are ions. —B_3: is a Lewis base (unshared pair of electrons) attached to a polymeric structure. —B_3: may share the pair of electrons with a cation: B_4^+, thus becoming converted to the charged form. Presumably, cation exchangers of the Lewis acid type can be prepared.

In laboratory use the organic exchangers have largely replaced the inorganic, being in general stable over a wider range of pH.

TABLE IX-2
Periodic Classification of Ion Exchangers and Possible Ion Exchangers (Incomplete)

Group	1	2	3	4	5	6	7	8
Period								
II				Ph—OH R—COOH	RNH_2 R_2NH R_3N R_4N^+			
III			"=Al—OH" Zeolites, clays, alumina	"—Si—OH" (with bonds above and below Si) Silicates, clays	RPH_2, etc. RPO_3H_2	RSH RSO_2H RSO_3H		
IV		"—CaOH"			$RAsO_3H$			$R—Fe^{++}$ $R—Fe^{+++}$
V								
VI		$R—Hg^+$						

Note: R is an organic resin backbone, often aromatic in nature; Ph is aromatic, that is, Ph—OH is a phenolic exchange site.

III. ION EXCHANGE MATERIALS

1. Functional Types

A. INORGANIC CATION EXCHANGERS

Inorganic cation exchangers comprise the zeolites, many clays, and some synthetic zeolites, and alumina. There are a large number of zeolite minerals—over thirty. A great variety of clay minerals, some of which show exchange capacity, are known (14), and the natural zeolites seem to belong in this group. Many of these substances are used com-

mercially as exchangers, principally in water-softening applications. Among the natural inorganic substances so used are greensands (glauconite) processed in various ways, Liquonex C G, Liquonex C G H, Verdite, and Zeo-Dur; and Refinite Z N, a processed bentonitic clay.

The synthetic zeolites are alumino-silicates, usually prepared in the sodium form: Crystalite, Decalso, Doucil, Liquonex CSL, Nalcolite, Permutit, R-P Zeolite. Alumina shows some cation exchange capacity when it has been treated with base (836), though it is rather low—of the order of 0.01 meq./g.

The exchanger sites in inorganic cation exchangers seem to be of the type $\equiv \mathrm{Si} - \mathrm{O}^- M^+$, or $= \mathrm{Al} - \mathrm{O}^- M^+$, though others may be present.

B. INORGANIC ANION EXCHANGERS

Hydroxy apatite has been studied as an anion exchanger for removal of fluoride from water (154). In laboratory applications alumina has been converted to an anion exchanger by washing with acid (508). Its capacity is, however, low.

Inorganic exchangers together with their characteristics and sources are listed in Table IX-3.

TABLE IX-3
Inorganic Cation Exchange Materials
Names, Compositions, Capacities, and Sources

National Aluminate Corp.
Nalcolite, a synthetic zeolite
The Permutit Company
Decalso, an alumino-silicate gel
Zeo-Dur, a processed greensand
Some clays show exchange capacity. Bentonite, *ca.* 1 meq./g.; montmorillonite, *ca.* 1 meq./g.

C. ORGANIC CATION EXCHANGERS

Organic cation exchangers may be classified in terms of the polymeric backbone and in terms of the type of fixed anion. The polymeric backbone may be a modified natural product, such as *coal* that has been sulfonated or oxidized; or cellulose that has been esterified with one function of a dibasic acid (584). It may be a synthetic polymer of the phenoplast type chemically modified by sulfonation or other treatment, or unmodified, or of the polystyrene type; or presumably of a variety of other addition or condensation types of polymer. The organic cation exchanger may also be a purely naturally occurring material (199). The

type of cationic groups responsible for the exchange ($R—A^-$) may be one or a mixture of the following: phenolic, carboxylic, thiol, sulfinic, sulfonic, phosphonous, phosphonic (107), arsonic, and presumably others that follow this pattern.

D. ORGANIC ANION EXCHANGERS

Organic anion exchangers can be classed in the same arguments as the cation exchangers. The backbone polymers fall in the same categories except that coal does not seem to have been utilized as a starting material. The types of groups responsible for the exchange may be Lewis bases ($R—B:$) such as $R—\ddot{N}H_2$, $R—\ddot{N}HR'$, $R—\ddot{N}R'R''$, where R′ and R″ may be the same or different groups, such as methyl or ethyl, or where the $—\ddot{N}=$ may be part of a ring, as in a pyridine polymer. The anion exchanger groups may also be permanent cations of the type $R—\dot{N}R'R''R'''$, where N is quaternized, and R′, R″, R‴ may be the same or different. Anion exchangers with other basic groups following this pattern are presumably possible.

Organic ion exchangers, their characteristics and sources, are listed in Table IX-4. (One of the difficulties in preparing such listings is that distributors will purchase exchangers from a primary manufacturer and sell them under their own name (possibly having them prepared to certain specifications). These names may be changed for reasons unconnected with exchange behaviors, and a listing becomes out of date as a consequence.)

2. Degree of Cross-Linking

The degree of cross-linking in ion exchange polymers controls two important properties of the polymers: the degree of swelling in water and the accessibility of the interior of the polymer to ions of different sizes. The degree of swelling is also affected by the "concentration" of fixed exchange sites in the resin. The degree of cross-linking is conveniently described in the case of polystyrene base resins where, also, control over degree of cross-linking is feasible in terms of the per cent of divinyl benzene (DVB) used in cross-linking. A 1% to 2% or lower degree of cross-linking represents a light degree; the ordinary commercial resins are about 8% cross-linked. The ultimate in cross-linked resin, poly-DVB, has been prepared (345).

Samuelson has given a thorough discussion of these behaviors (803). He states that certain exchangers can be prepared which are chemically quite stable yet which "swell in water to a volume of 100 ml. or more

TABLE IX-4

Organic Ion Exchange Resins.[a] Names, Compositions, Capacities[b] and Sources
(See Appendix for addresses of the source firms)

Chemical Process Company (Duolite & Chempro.[c])

Anion exchangers

Weak base. Duolite A-2 (8.4 meq./g.); A-4 (7.7); A-5 (8.5); A-6 (7.0); A-7 (9.1); A-10 (9.1). Intermediate base. A-30 (8.7); A-70 (7.2). Strong base (quaternary type). A-40 (3.7); A-42 (2.3); A-44 (2.6); A-41 (8.0 acid absorption, 2.8 salt-splitting); A-43 (8.0 and 2.5, as above); A-47 (7.5 and 2.6, as above).

Cation exchangers

Sulfonic acid type. Phenolic base. Duolite C-3 (2.9); C-10 (2.9). Styrene base. Chempro C-20 (4.9); C-21; Duolite C-25 (4.6); C-25L. Carboxylic acid type. Duolite CS-100 (1.9); CS-101 (8.3). Phosphorus-containing types. Duolite C-60 (6.0) phosphonous acid type; C-61 (6.6) phosphonic acid type; C-65 (3.3) phosphonic acid type.

Zwitterion resin.

The Dow Chemical Company (Dowex[c])

Anion exchangers

Weak base: Dowex 3 (6 meq./g.) polyalkylamine groups on styrene-divinyl benzene (DVB) copolymer matrix. Strong base: Dowex 1 (3.5) quaternary ammonium groups on styrene-DVB matrix; 2 (3.5), same type as Dowex 1, except one of the alkyl groups on the nitrogen is β-hydroxy ethyl.

Cation exchangers

Dowex 50 (5 meq./g.) nuclear sulfonic acid groups on styrene DVB matrix. 50W, light-colored form of Dowex 50.

These resins can be obtained with a variety of porosities, controlled by the per cent DVB cross-linking agent, from 1 to *ca.* 16% DVB. A lightly cross-linked resin might be permeable to ions not able to enter a resin with a higher per cent of cross-linking agent. The designation of cross-linking is indicated thus: Dowex 50-X4 is Dowex 50 containing 4% DVB cross-linking agent.

National Aluminate Corporation (Nalcite[c])

Anion exchangers (The numbers after the names indicate per cent DVB used)

Weak base: Nalcite WBR, polyamine groups on styrene-DVB matrix. Strong base: (quaternary groups) SBR-1; SBR-2; SBR-4; SBR-7.5; SBR-8; SBR-10; SAR-4; SAR-7.5; SAR-8; SAR-10.

Cation exchangers (Nuclear sulfonic types)

HCR-1; HCR-2; HCR-4; HCR-5; HCR-8; HCR-10; HCR-12; HCR-16; HCR-W-8; HCR-W-12. All the above resins are made for National Aluminate Corp. by the Dow Chemical Company.

The Permutit Company

Anion exchangers

Weak base: De-Acidite[c] (*ca.* 9.3 meq./g.) (aliphatic polyamine). Strong base: Permutit[c] S 1; Permutit S-2 (quaternary amine polystyrene)

Cation exchangers

Permutit[c] Q (sulfonated polystyrene); Zeo Karb[c] (sulfonated organic coal derivative).

Rohm and Haas Company, The Resinous Products Division (Amberlite)[c]

Anion exchangers

Weak base: Amberlite IR-45. Strong base: IRA-400 (ca. 2.5) (quaternary amine type); IRA-401 (amine-type resin); IRA-410 (quaternary amine type).

TABLE IX-4 (*Continued*)

Organic Ion Exchange Resins[a] Names, Compositions, Capacities[b] and Sources
(See Appendix for addresses of the source firms)

Cation exchangers
Amberlite IR-120 (4.2) (nuclear sulfonic acid type); IRC-50 (10.0) (carboxylic type).
Resinous Products are making available through Fisher Scientific Company, chromatographic grade exchangers which are designated CG- with the above numbers replacing IR-, IRA-, and IRC. Most laboratory supply houses handle ion exchange resins.

[a] Chemical Products Company produces Duolite Electron Exchanger, S-10, a copper-amine complex resin (see Chapter X).
[b] Given in milliequivalents per gram of dry resin, after the name or number of the resin.
[c] T.M., registered.
Note: Many of the exchangers now produced (May, 1956) are subject to improvement or other changes. New exchangers will certainly become available. This table cannot therefore be expected to be other than a convenient point of departure for the reader.

calculated on 1 g. of dried resin." The ordinary commercial exchangers do not swell so much, but may have swelled volumes of 2 to 3 ml. per gram of dried resin.

For practical purposes, resins that swell or shrink greatly when the concentration of solution passing over them decreases or increases in ionic strength are not desired because of difficulties with apparatus. Glass tubes containing beds of resin have been shattered by expansion of the bed, or the bed channels have been closed down to such small diameter that flow has been impeded or stopped. Yet if the resin is so highly cross-linked that it virtually does not undergo swelling or shrinkage, it is likely to be of greatly decreased usefulness because of inaccessibility of the interior and consequent lowered capacity. A balance between these two is chosen in commercial exchangers, and most apparatus for ion exchange chromatography is designed so that the bed may be backwashed, so that liquid may be forced up through the bed, suspending the particles enough that they may freely swell during a cycle of the operation.

There are applications in which the degree of cross-linking of the resin plays a required role, either in excluding or admitting certain molecules. Samuelson has emphasized that from the analytical point of view it is important that ordinary exchangers do not retain colloids and even high molecular weight ions. This permits separations that would otherwise by very difficult (803).

On the other hand, in the separation of large molecules (ionic or nonionic), exchangers with lower degrees of cross-linking are necessary (190) so that the large molecules may enter the body of the resin and be sorted on the basis of their specific interactions with the resin surface.

Resins with different degrees of cross-linking are now available; see Table IX-4.

IV. EXCHANGE BEHAVIOR

1. Qualitative Considerations

The relative position of the exchange equilibrium for two exchangeable ions in a solution in equilibrium with an exchanger is a measure of the ability of the exchanger to differentiate the ions: to take one up more than the other. This is measured by a *separation factor* (469), which may be defined in various ways to suit the needs of the occasion, that is, in terms of concentrations, activities, or ratios of equilibrium constants (106,327,345,620,676,803). The separation factor K_s may be defined as:

$$K_s = (X_2/X_1)(C_1/C_2)$$

where X_2 and X_1 are mole fractions of the two ions in the resin phase and C_2 and C_1 the corresponding concentrations outside the resin phase. From this it can be seen that the further this ratio is from 1 in a given system the easier it will be to separate the ionic species. When this ratio is of the order of 1.2, the species should be separable efficiently by chromatography. It is important to take into account, in determining selectivity, the imbibition of the resin by the solution.

Studies on selectivity of cation exchangers indicate that some (106) of the factors that may influence it (equilibrium conditions, aqueous solutions, univalent cations) are structure and capacity of the resin, nature of the functional group ($—A_1^-$) mole fractions of exchanging cations inside the resin, ionic strength of ambient solution, and temperature. The effects of these factors may be summarized from Bregman (106), who refers to the literature.

At the degree of cross-linking of about 8% the order of selectivity is $K^+ > NH_4^+ > Na^+ > H^+ > Li^+$ (345). With decrease in cross-linking, selectivity decreases, and all the ions seem to "look alike" to the resin, except the ammonium ion, which becomes preferred to potassium below about 2% cross-linking. As the cross-linking is increased above around 8 to 16, selectivity decreases until when the high value of 26% is reached lithium is preferred to potassium. At least part of this reversal is laid to the lower effective hydrated size of the lithium ion, compared to the potassium within the resin phase where the molality is of the order of 10 molal. The selectivity of one type of resin changed with the capacity at a given degree of cross-linking (106,345).

The kind of functional group has been known to affect selectivity. Bregman (106) has compiled the following examples: with a sulfonic

resin, $K^+ > Na^+ > Li^+$; with a carboxylic resin and a phosphonic resin, $Li^+ > Na^+ > K^+$. These effects are relatable to relative polarizabilities of the groups $(-A_1^-)$ in the resin and to other properties of the systems. Thus the order of decreasing solubilities is related to the order of increasing resin affinities, among other correlations noted.

The affinity of a resin for one cation compared with that for another seems to increase as the mole fraction of that ion decreases inside the resin, but remains fairly constant as the ionic strength outside the resin is changed over a range of low concentrations (106).

Behavior of anion exchangers has been reviewed by Peterson (728), among others (48,676,803,829).

A number of collections have been made of relative affinities which suggest what separations between the ions may be made. Some of these are gathered in Table IX-5.

TABLE IX-5
Some Relative Affinities of Ions

Type of ion	Exchangers
	Sulfonic acid-type (strong-acid)
M^+ (49,498,803)	$(CH_3)_4N < Li < H < Na < NH_4 < K < Rb < Cs < Tl < Ag$
M^{+2} (996)	$Mg < Ca < Sr < Ba$
M^{+3} (57)	$Al < Sc < Y < Eu < Sm < Nd < Pr < Ce < La$
M (469)	$Lu < Yb < Tm < Er < Ho < Y < Dy < Tb < Gd < Eu < Sm < Pm < Nd < Pr < Ce < La$
	Carboxylic acid (weak-acid type)
M^+ (106)	$K < Na < Li$
	Quaternary ammonium (strong-base type)
A^-, some $A^=$ (510,988)	acetate < fluoride < hydroxyl < formate < chloride < thiocyanate < bromide < chromate < nitrate < iodide < oxalate < sulfate < citrate (The order may change to some extent with concentration.)
	Amine (weak-base type)
A (511)	fluoride < chloride < bromide = iodide = acetate < molybdate < phosphate < arsenate < nitrate < tartrate < citrate < chromate < sulfate < hydroxyl (The order may change to some extent with concentration.)

2. Quantitative Considerations

Ion exchangers behave as adsorbents in the sense that, as they are insoluble substances, the exchange can occur only at the "surface" of the solid in contact with the solution. It is very probable that with some exchangers the polymer structure is so open that one can speak of a sur-

face with any meaning only if it is understood that the word is used in an extreme sense, that is, in the sense used by Langmuir when he wrote of the "surface" of a molecule (521). In any event, to say that ion exchangers act by exchange adsorption is not to imply a mechanism. There seems to be some misunderstanding of this in the literature, and perhaps it would be better to drop the term adsorption in this application.

The exchange reaction can be represented *in terms of* an adsorption isotherm, with its attendant equilibrium treatment of the reaction:

$$\text{substance in interface} \rightleftharpoons \text{substance in solution}$$

A plot of the relation between the amount adsorbed per unit weight of adsorbent and the concentration in a fixed volume of solution in equilibrium with this at a given temperature yields an adsorption isotherm of the usual form (101,450,451,606,971). It should be noted that usually in this method of plotting only one of the exchanging ions is explicitly accounted for; the other ion is probably not forgotten, but is neglected. This kind of treatment is legitimate if the concentration of the ion being adsorbed, say M^+, is quite low compared to that of the other ion, so that when M^+ is adsorbed the amount of the other ion which is displaced is not enough to alter the concentration appreciably or to change the amount of liquid in the resin phase (see below). If there is much change in volume of the resin this must be taken into account as it withdraws liquid and ions from the outside phase. This situation has been encountered before.

The adsorption, or exchange, process can also be investigated *in terms of* the rates of the two reactions involved in the exchange of an ion:

$$\mathrm{R}^-M_1{}^+ + M_2{}^+ \rightleftharpoons \mathrm{R}^-M_2{}^+ + M_1{}^+$$

The forward and the reverse reaction velocities can be examined and the kinetics of the process analyzed (676,803,829,960). Such an analysis may lead to conclusions about the mechanism of the exchange. For example, Boyd and co-workers (100) (see below) have found conditions under which the rate of an exchange reaction is controlled by the velocity of diffusion inside the resin particle, and conditions under which diffusion through the immobile film about the particle is rate-determining.

The exchange of ions has been treated by Mayer and Tompkins (620) *in terms of* a distribution process analogous to distillation. A distribution coefficient could be derived for the exchange, and mass law relationships applied. *In other terms*, the insoluble resin particle could be looked upon as participating in a Donnan type of partition with the ambient solution as the other phase (49,217,939). These kinds of treatment will be discussed briefly below.

In a typical exchange experiment the solution is analyzed before addition of the exchanger and after equilibrium has been reached, the difference between the two analyses being taken to represent the amount of substance adsorbed or sorbed. This is plotted in the usual way against the appropriate equilibrium concentration. The time required to reach equilibrium varies considerably with different types of resins and different activations. In the adsorption of arginine on a cation resin activated with 5% hydrochloric acid equilibrium was reached in about 1 hr. But when the resin was activated with sodium carbonate the exchange seemed to continue for at least 36 hr., though the possibility of destruction of the amino acid, which would simulate adsorption, was not excluded (185). Similar behavior has been observed in other connections (675). The rate of approach to equilibrium is not necessarily related to the capacity of the exchanger.

The data obtained from such an experiment may be expressed in terms of the Langmuir isotherm or of the Freundlich isotherm (Chapter IV). The Langmuir isotherm has been used to fit exchange data by many experimenters; however, a modification in the concept on which the isotherm was derived is required (101). There are few or no "bare spots" on the surface of the exchanger (otherwise the particles of the adsorbent would be found to be charged), but instead each site at which exchange may occur has associated with it either the type of ion originally present in the resin or an ion obtained by exchange from the solution. The equation is written, neglecting any "bare" surface:

$$\left(\frac{x}{m}\right)_{A^+} = \frac{kb_1C_{A+}}{b_1C_{A+} + b_2C_{B+}} = \frac{k\,(b_1/b_2)(C_{A+}/C_{B+})}{1 + (b_1/b_2)(C_{A+}/C_{B+})}$$

where $(x/m)_{A^+}$ is the quantity of ion A^+ adsorbed per unit weight of adsorbent; C_{A^+} and C_{B^+} are the respective equilibrium concentrations or activities of the ions A^+ and B^+ in solution; and b_1, b_2, and k are constants. Boyd and co-workers point out that according to this equation the amount of A^+ adsorbed must depend on the ratio of the concentrations of the ions A^+ and B^+ in solution at equilibrium and hence will be independent of the dilution (as is found by the authors for the adsorption of a number of cations on an organic exchanger). They point out also that for the case where C_{B^+} is very much larger than C_{A^+} the adsorption of A^+ becomes approximately directly proportional to C_{A^+}, whereas for C_{A^+} very much larger than C_{B^+} the adsorption of A^+ is nearly constant and independent of concentration. The application of the Langmuir equation is discussed and generalized by these authors. They point out that the treatment depends upon the assumptions that only one kind of exchange site is present in the surface (i.e., a single type of functional

group), that there is no interaction between adsorbed ions, and that no double layers are formed (i.e., an adsorbed cation would not then take up an anion from solution and this another cation). It might be mentioned, in passing, that even a single functional group type may present an effectively heterogeneous exchange surface, since the relative locations of the groups—their geometry on the surface—may influence their exchange potentials.

A number of experimental and theoretical investigations have been made into the kinetic aspects of the exchange process. An example can be taken from the work of Juda and Carron (456). These authors studied the exchange of hydrogen ion and sodium ion on four organic resins. This exchange involved the reaction:

$$R^-H^+ + Na^+ \rightleftharpoons R^-Na^+ + H^+$$

which was examined starting with the acid form of the resin $R^- H^+$ and treating with $Na^+ Cl^-$, or starting with the sodium form $R^- Na^+$ and treating with $H^+ Cl^-$ solution. The concentration of hydrogen ions in the aqueous phase was determined experimentally; then, knowing the number of hydrogen ions and sodium ions initially available, the ratio in the exchanger phase could be deduced, assuming the ionic strength of the water phase to remain constant throughout the exchange. The equilibrium constant of the reaction:

$$\frac{C_{Na^+}\text{ (on resin)} \times C_{H^+}\text{ (in solution)}}{C_{H^+}\text{ (on resin)} \times C_{Na^+}\text{ (in solution)}} = K$$

was worked out on the basis of concentrations and activities; also the velocity constants for the two opposing reactions were worked out. Since the concentrations of sodium and hydrogen ions in the exchanger were not readily accessible, *relative* amounts of exchanger and solution phases were connected to the rate constants, giving a relationship which was subject to experimental test (940).

Certain simplifying assumptions and arrangements were made in designing the theory and experiment. Interionic effects in the exchanger and solution were ignored. It was assumed that the hydrogen ion concentration in the original sodium chloride solutions before contact with the acid form of the resin was zero, and that in the sodium form of the resin used at the start of the reverse reaction it was also zero. It was assumed that the hydrochloric acid used in the reverse reaction was completely ionized. In preparing the acid form of the resins it was found that drying at 80 to 85°C. released acid, presumably through decomposition of the resin; therefore the resins were air-dried to constant weight, and corrections (usually small) made where necessary for such hydrogen ions as

were leachable from the prepared resins by distilled water. The sodium form of the resin was also air-dried. Measurements of pH and chloride indicated that the washed, prepared resins did not release sodium ions to distilled water. The exchange capacities were determined on the basis of bone-dry resin to avoid the variable amounts of water present in air-dry material; and here, because of the decompositions observed on heating the acid form of the resin, a definite temperature and duration of drying (85°C. for 24 hr.) was used, with a sample of 20/40 mesh particle size. Corrections for residual hydrogen ion in the sodium resin and sodium ion in the hydrogen resin were applied when the errors introduced by their neglect would affect the equilibrium constant by 10% or

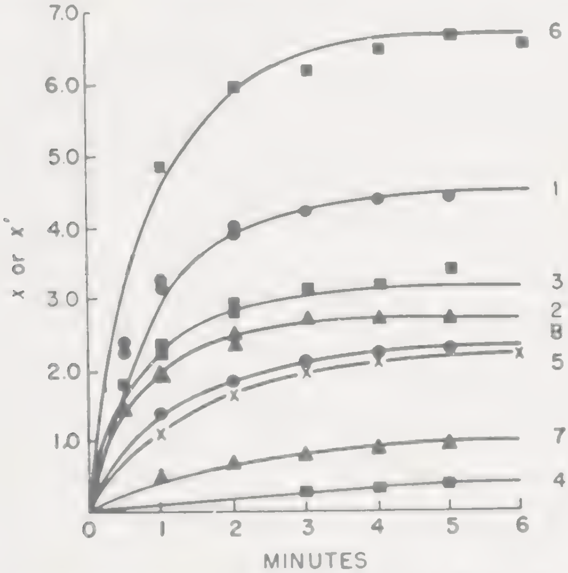


Fig. IX-1. Rate experiments on the exchange of H^+ and Na^+ on Zeo-Karb. The numbers of the curves correspond to the numbered runs in Table IX-6. x is the hydrogen ion concentration in solution, milliequivalents per liter, at the time t for the reaction $R^-H^+ + Na^+ \rightarrow R^-Na^+ + H^+$. x' is the sodium ion concentration in solution at time t for the reverse reaction. (From Juda and Carron (456).)

more. It was assumed that the volume of the wet exchanger phase was independent of the relative amount of sodium and hydrogen ions present in the resin—an assumption which seemed to be reasonable in this case. It was observed that the reproducibility of the data for the runs in which R^-Na^+ was reacted with H^+ were not so good as for the reverse reaction. This was laid to an attack on the resin by the concentrated salt solutions used to prepare the sodium forms. It was found unnecessary to control the temperature more closely than to 28 ± 3°. Figure IX-1 and Table IX-6 show the results of one set of experiments.

Results of the experiments on the four exchangers agreed reasonably well with a simple second-order rate equation for opposing simultaneous reversible reactions. In individual runs k and k' (Table IX-6) were usually constant to better than 20%, and were of the same order of magnitude for all four exchangers, but the values of k and k' increased with decrease in concentration—an effect laid to the dependence of the k values on the ionic strength both inside and outside the resin particle. The ratio k/k' also agreed well with the equilibrium constant K determined separately. These measurements of Juda and Carron support the concept of the wet exchanger as a phase, containing available ionized hydrogen ions which are able to diffuse to a small distance from the fixed anionic groups, and which can be displaced by other cations, the forward and reverse reactions appearing as a reversible, second-order reaction.

TABLE IX-6

Data on Rates for the Exchange of H^+ and Na^+ on Zeo-Karb

Forward Reaction: R—H^+ + Na^+					
Run No.	a	b	x_e	k	$k_{av.}$
4	1.16	1.76	0.62	0.061–0.066	0.062
5	4.07	7.42	2.35	0.044–0.045	0.045
1	9.25	11.46	4.55	0.039–0.043	0.041
6	15.80	15.32	6.77	0.028–0.030	0.029
2	20.68	3.67	2.80	0.038–0.044	0.042
Reverse Reaction: R—Na^+ + H^+					
Run No.	c	d	x'	k'	$k'_{av.}$
7	1.95	2.39	1.04	0.10 –0.13	0.110
8	6.01	3.82	2.29	0.064–0.083	0.071
3	9.40	3.92	3.20	0.110–0.127	0.118

Here a = initial sodium ion concentration, in meq./l.; $b = 1000(w_{H^+}M)/v_{Na^+}$, where w_{H^+} is the initial weight in grams of the hydrogen form of the resin containing M milliequivalents of exchangeable ions per gram in contact with v_{Na^+} cc. of the sodium chloride solution of concentration, a; x_e = the milliequivalents of hydrogen ion exchanged at equilibrium; k = the velocity constant for the forward reaction in liters per milliequivalent per minute. c, d, x', and k' have the corresponding values for the reverse reaction, c being the initial concentration of hydrochloric acid in milliequivalents per liter.

The mechanism of such an exchange involves diffusion of the ions inside the particle of wetted resin, diffusion through the interface between the resin phase and the solution, and the actual chemical exchange process. The mechanism was analyzed by Boyd, Adamson, and Myers (100) in order to discover which steps were rate-controlling and under what conditions. The phenomena here are similar to those encountered in other distribution processes (Chapter VI), where diffusion across a stationary

film diffusion within the two phases in contact, physical interactions and (possibly) chemical reaction are steps in the process of the distribution.

Boyd and co-workers (100) studied the exchange of a number of alkali metal cations on the resin Amberlite IR-1, using a tracer technique (radioactive sodium, rubidium, and cesium) which permitted exchange from dilute solutions of the exchanging ion. Thus in the reaction:

$$R^-B^+ + A^+ \rightleftharpoons R^- A^+ + B^+$$

if A^+ is present in concentration much lower than B^+, then the composition of the exchanger could be taken as essentially unchanged by the (slow) adsorption of A^+, and the small amount of B^+ displaced would not, up to a point, seriously change the (high) concentration of B^+ in the solution. An improved experimental method was used which enabled the rate data to be determined under conditions of effectively constant solution concentration. This was accomplished by flowing the test solution through a thin bed (254) of the exchanger for a determined time, whereupon the exchanger was rinsed and dried with acetone, and the amount of tracer in the solid was estimated by standard counting procedures.

Of the steps in the distribution process mentioned above, diffusion up to the particle boundary was eliminated as a possible rate-controlling factor by the good contact of constant composition solution with the exchanger particles obtained in the flow process. There remained to be examined the steps: diffusion of the cations and anions of the solution through the stationary interfacial film (430); diffusion within the particle, either in channels or along the pore walls; exchange reaction at the exchange sites within the particles; diffusion of displaced cation, with anion, back out of the particle; diffusion back through the film surrounding the particle. The last two steps are the reverse of the first two.

Equations were set up to describe the rate of each of the steps: diffusion through the stationary film bounding the particles; diffusion through the exchanger particle, with constant solution concentration; and chemical exchange (governed by mass law). It was found that the rate equations for diffusion through the stationary film and for the chemical exchange were of the same *form* and different from the equations for diffusion in the particle. Experimental determinations of the rates of exchange were tested for conformity with the equations. An example of the results is shown in Fig. IX-2. The curves represent the velocities of uptake of sodium ion from 0.1 and 0.001 *M* KCl solutions containing 8×10^{-5} *M* NaCl. Evidently, in solutions of 0.1 *M* (or greater) concentration the diffusion within the exchanger particle is the rate-controlling process; in solutions of 0.001 *M* (or lower) the rate of "exchange" is controlled by

the diffusion through the film enveloping the exchanger particle. Further analysis of the exchange process indicated that the diffusion constants for ions in the exchanger particle are from five- to tenfold smaller in magnitude than for the same ions in aqueous solution. Bauman and Eichorn (49) found a fivefold difference for diffusion of NaCl and HCl in Dowex-50 spheres compared with water. It was observed also that the diffusion of an ion present in small amount in solution with a large amount of another ion is speeded up if the latter has a larger diffusion constant, indicating ionic interaction.

The implications of these and other findings are discussed by the authors. They were able to distinguish whether chemical exchange or the

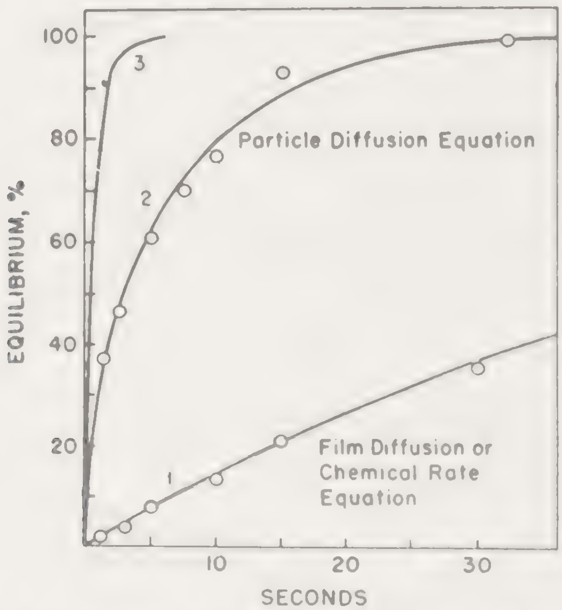


Fig. IX-2. Rate of exchange of sodium ion at 30°C. from 0.001 M (curve 1) and 0.1 M (curve 2) aqueous potassium chloride solutions by 60/70 mesh Amberlite IR-1. The solutions were 8×10^{-6} M in NaCl. The solid lines represent the best-fitting theoretical curves. Curve 3 is the curve which should be fitted if boundary film diffusion (or chemical exchange) were the rate-controlling step in the 0.1 M solution of potassium chloride. (From Boyd, Adamson, and L. S. Myers, Jr. (100).)

boundary film diffusion was the rate-controlling step in the dilute solutions by an analysis of the shapes taken by the experimental curves when particle size or film thickness (controlled by flow rate) were changed (which would affect the film-diffusion step) or when concentration and temperature (which would affect the chemical exchange rate) were changed. It was concluded that the rate of exchange was diffusion controlled.

The Donnan concept (916) has been applied to the exchange process by a number of workers (48,49,217,343–345,348,803). The application is based on the idea that the wet resin body in contact with solution forms a heterogeneous system between the two phases of which mobile ions can migrate but in one phase of which a nondiffusible ion exists. The situation can be shown in generalized form thus:

Resin phase	*Solution phase*
$R^- \quad M_1^+ \; A^- \; M_2^+$	$M_1^+ \; A^- \; M_2^+$

According to the Donnan concept, in terms of molar activities (a), at equilibrium:

$$a_{M_1^+}a_{A^-} \text{ (in resin)} = a_{M_1^+}a_{A^-} \text{ (in solution)}$$

and:

$$a_{M_2^+}a_{A^-} \text{ (in resin)} = a_{M_2^+}a_{A^-} \text{ (in solution)}$$

or:

$$\frac{a_{M_1^+} \text{ (in resin)}}{a_{M_1^+} \text{ (in solution)}} = \frac{a_{M_2^+} \text{ (in resin)}}{a_{M_2^+} \text{ (in solution)}}$$

For dilute solutions the equations can be written in terms of concentration (C = molar concentration):

$$\frac{C_{M_1^+} \text{ (resin)} \times C_{M_2^+} \text{ (solution)}}{C_{M_1^+} \text{ (solution)} \times C_{M_2^+} \text{ (resin)}} = K_{equil.}$$

Bauman and Eichorn tested this relation for the case of diffusion of HCl into water-wet Dowex-50 (sulfonic acid resin $RSO_3^-\ M^+$) in the acid form. There was no exchange, merely diffusion of $H^+\ Cl^-$ into the resin. The resin decreased in volume with increase in the equilibrium concentration of acid. The concentration of HCl inside the resin particles was always lower than the equilibrium concentration outside. The Donnan relation was followed well at the higher HCl concentrations. The exchange equilibrium was also tested against the Donnan relation by immersing the hydrogen form of the resin in solutions of various cations at different normalities. The equilibrium constant K (above) held well for normalities from 0.01 to 1.0 (for the ammonium–hydrogen ion exchange, for example) but in the concentrated solutions K decreased. The authors think it quite possible that the position of the ion exchange equilibrium in this resin is determined by the difference in activity coefficients between the solution phase and the highly concentrated resin phase without involving any specific affinity of sulfonate groups for one ion over another. The mass

law equilibrium relation held well also for exchange of a divalent ion on hydrogen exchanger. Rate studies led to equilibrium constants in good agreement with the $K_{equil.}$ determined by batchwise experiments.

These experiments support the conclusion that this resin (Dowex-50) when in contact with water is equivalent to a highly ionized strong salt solution. The exchanger in contact with a solution of diffusible ions takes these up to an extent and at a rate governed by the law of mass action. At equilibrium the concentration of the diffusible ions in the exchanger is then lower (Donnan effect) than that in the aqueous phase. The rate behavior suggests that for solutions of concentration above about 0.1 molal, diffusion within the resin particle should be the slowest, or rate-controlling, process. These data are also supported by the work of Juda and Carron (456).

Exchange substances have been treated as though they were solid solutions. Kielland (473) has employed this approach. The exchanger has been treated by Vanselow (960) as though it were a mixed crystal.

The fact that the data conform with a particular mathematical expression, derived on some reasonable hypothesis, perhaps with a number of simplifying suppositions, does not *necessarily* mean that the data are explained by the hypothesis and none other. This is, of course, recognized. For example, Juda and Carron (456) point out that in the exchanges which they studied a Donnan equilibrium concept (217) leads to an equilibrium relation of the same form as a base-exchange concept, and that therefore their experimental data are not adequate to make possible a decision between the two. In another case, cited above, Boyd and coworkers (100) found that a rate equation based on a chemical exchange mechanism gave an expression of the same form as an equation for the rate of diffusion through a thin film, so that no decision as to which was the rate-controlling step could be reached from a single set of rate experiments. However, in this case a decision was reached following a study of several factors which influenced the exchange, and this ruled out the chemical exchange as the rate-controlling step.

The factors that may complicate resin behavior are probably not all known or imagined. Some, however, may be listed. The existence of several types of functional groups at the interface would make the capacity of the exchanger dependent at least on pH if not also on specific interactions with ions. This is one of the interfacial factors which may complicate resin behavior. Probably all resins, whether chemically homogeneous or not, will on more subtle analysis show inhomogeneities related to the geometric arrangements of the functional groups. This might be so especially if several types of functional groups are present, when, for example, an *ortho* arrangement may permit a chelation which

a *meta* or *para* arrangement would not allow. Or it might be so for the exchange of a polyvalent ion, which might be more tightly held through the cooperation of several functional groups suitably spaced. Other interfacial factors such as porosity may be mentioned. Resins may differ in extent of internal structure, that is, in the ratio of large to small channels, and in the presence of tapering, irregular channels, of nonuniform diameters. The density of polar portions of the surface (the ratio of polar to nonpolar) may differ between organic resins and may not be uniform in a given resin.

As regards the resin phase, one factor that may be of importance in the exchange is the change in resin volume with change in pH of the solution, or with change in the nature or concentration of the ions present. There is also the possibility of change in particle size caused by cracking of particles under swelling and contraction changes or mechanical attrition, which might affect capacities (especially with the inorganic exchangers). Another factor might be the dissolution of organic molecules by the resin. The high densities of charge in some exchangers might certainly be expected to cause adsorbed ions and solvent to behave in a most unideal manner. The presence of occluded gases may prevent or hamper contact with the solution.

Factors outside the resin particle may also be effective. Among these would be the changes in size of ions (hydrated radius) with change in concentration or composition of the solution. The pH-dependent competitions for exchange sites by solvent ions (H^+ or OH^-) may in certain cases be important.

A rigorous thermodynamic approach to the calculation of cation exchange selectivities has been presented by G. E. Myers and G. E. Boyd (673a).

V. CATALYSIS

Many results of catalysis due to ion exchange resins have been published (895). A very extensive table containing over 80 examples has been given in the review of this subject by Helferich (382). The examples comprise hydrolyses of glycosides, esters, peptides, proteins caused by acid resins; saponifications by basic resins; condensations and addition reactions caused by acid: esterification, ether formation, acetal and enol ether formation; condensations and addition reactions caused by base: aldol condensations, Knoevenagel condensations, cyanoethylation, cyanhydrin and nitroalcohol formation; alcoholysis by acid resins; addition of amines by basic resins; addition of water to acetylenes by cation exchanger carrying mercury ion; water removal by acid catalysis; splitting out of

hydrochloric acid by a basic resin; rearrangements by acid as well as basic resins. The theory of these reactions has been approached by Helferich (383), but a discussion of this topic will not be given here (67,574, 779).

VI. ION EXCLUSION AND ION RETARDATION

Wheaton and Bauman (989,990) have developed a technique called ion exclusion that relies on the fact that the concentration of an ionic species inside a resin particle is markedly different from that in the solution outside the particle, whereas the concentration of small nonionic species is not markedly different. The concentration of ionic species, the fixed charged groups, in the resin particle, is normally higher than in the outside medium, and with ordinary ion exchange resins may reach high values as 2 to 10 N. This phenomenon had been noted by Jenny in connection with permutits (450).

Because of the high ionic concentration within the particle, ionic species outside of it would be excluded—quite effectively in dilute solution. If, for example, the resin were in the form $RSO_3^- Na^+$, then all sodium salts would be excluded. However, potassium salts would exchange potassium for sodium to some extent, though the anions might be largely excluded.

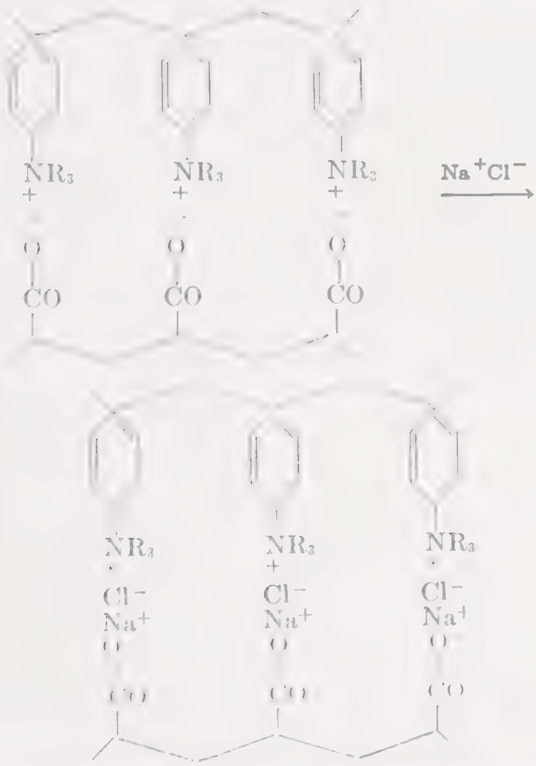
But the high ionic strength inside the particle, compared with that outside, has little effect on nonionic species of molecules provided that they do not react with the polymer. If such molecules are small enough to diffuse into the resin with the particular degree of cross-linking in use, then the resin will sorb these molecules to some extent, and *in a chromatographic arrangement these non-ionic species, since they interact with the stationary phase, will be retarded*, while the ionic species, and any nonionic molecules too large to enter the resin network, will move ahead. A separation will then occur. (See below among the examples of methods.)

In general, the greater the ionic strength in the mobile phase, the less sharp the exclusion of ions from the resin phase. The separation is improved by using smaller resin particles, probably because of improved approach to equilibrium. The elution curves are sharper for lower cross-linkages (990), though the separation is not so good, becoming zero for a completely un-cross-linked, soluble, polymer.

Ion retardation is the descriptive name given to a new technique that complements ion exclusion. A specially designed ion-exchange system is used (374a). This system comprises an ion-exchange resin with functional groups of one type such as strong base, weak base, strong acid, or weak acid, fixed to the polymer matrix. Within the pores of this resin, "like a snake in a cage" is polymerized a more or less linear polyelectrolyte of an

opposite type. Thus if the matrix were a Dowex-2 (dimethyl β-hydroxyethyl ammonium) type, the linear polyelectrolyte might be a polystyrenesulfonic acid or a polyacrylic acid. The acid (or base) groups on the linear polymer would interact with the base (or acid) group of the cage, forming an exchanger of mixed type, with the mixing at a molecular level.

When a solution containing exchangeable ions is placed in contact with this material (prepared with a cross-linked bead-form matrix), the ions, diffusing into the bead, can uncouple the linear polymer functional links from the matrix functional groups, neutralizing *both* to the extent that the selectivity relations allow. Thus if one had, for example, a polyacrylic acid (PAA) chain in the Dowex-2 cage and $Na^{+}Cl^{-}$ was introduced, the Cl^{-} would readily largely displace carboxylate from the ammonium nitrogen, since the selectivity operates in favor of chloride (Table IX-5), while the sodium would neutralize the freed carboxylate (this is a crude statement of the complex dynamic reaction):



The reaction is facilitated because the chain is flexible enough to allow the groups to move apart under the "prising" action of the solute. The resin would thus sorb an ionic substance that would, so to speak, "unzip" the chain from the matrix. Such a sorbed material can be washed out with water, when the external concentration is reduced. But if an ionic and a

nonionic substance, or an ionic and a less selectively preferred ionic substance were passed through a bed of resin, in both cases the former would be sorbed to a greater extent than the other and, entering the stationary resin phase, would be retarded. The result, in terms of effluent pattern would look like that in Fig. IX-5, except that here the alcohol zone would appear first and the salt zone would be retarded.

The special features of these resins cannot be discussed here, but may be found in the forthcoming scientific and industrial literature. Their behavior is affected by pH and by the other factors that affect ionic reactions. Mixtures of salts with a common anion or cation can be separated on the basis of selectivities, and, when mixed salts with different anions as well as cations are passed into bed of resin, the more strongly selected anions *and* cations are retarded more, so that metathetical reactions may result.

VII. SORPTION AND RELATED EFFECTS

Many authors have observed that ion exchangers may sorb nonionic species. Indeed, this is quite reasonably correlated with the highly developed porosity of these substances. Many also have large accessible surface area because of their porosity, and so these exchangers may show behaviors including nonexchange adsorptions, occlusions, and dissolutions, as well as actual chemical reactions. Nonexchange adsorptions are those in which the resin takes up on its surface neutral, nonionizing molecules. Such adsorptions are often difficult to distinguish from occlusions and dissolutions, and perhaps all three types of behavior should be treated under the heading of sorption. However, the appearance of a difference will be preserved here for the sake of emphasizing its possible existence.

Occlusion by zeolites has been the subject of numerous studies (28–31, 575,576). The term *occlusion* is used to describe a filling up of the pores of the zeolite by solvent and solute molecules, usually without the occurrence of exchange. Thus the very porous zeolites can take up large quantities of gases. Barrer has divided these zeolites into three classes on the basis of their sieve properties, i.e., their abilities to exclude molecules with a given or larger critical cross section and to occlude molecules with smaller cross sections. Synthetic molecular sieves are now available in a variety of pore sizes from Linde Air Products.

The occlusion, or molecular sieve, properties of zeolites were utilized by Barrer to separate mixtures of gases and of liquids. For example, in one experiment 2.0 ml. of a mixture of 20.7% *n*-heptane and 79.3% toluene was exposed to 6.0 g. chabazite in a sealed tube at 200°C. for 48 hr. The *n*-heptane was quantitatively removed from the mixture by occlu-

sion in the zeolite, while of 1.586 ml. toluene originally present 1.560 ml. was recovered. A number of such separations have been listed by Barrer and his co-workers, the applications involving such separations as ethane from propane in a flowing system at room temperature; and polar molecules from their mixtures. It is suggested that these zeolites may be used to break azeotropes, to dry organic vapors or liquids, and to remove small amounts of unwanted components from mixtures which have been partially purified by other processes. The utilization of the zeolites showed certain limitations, such as difficulties of recovery of the occluded substances in certain cases, polymerizations, and other reactions on, and in, the zeolite.

Organic polymers give some evidence of taking up certain substances by a process of dissolution in the body of the polymer. An example of this was noticed by Cleaver (183). He found that a number of amino acids were sorbed by cation exchange materials at pH values at which very small amounts of the amino acids could be in the cationic form; furthermore, amino acids with essentially the same isoelectric point were taken up to different extents, whence the conclusion seemed to be that the sorption was not of an ionic type. Englis and Fiess (270) had reported adsorptions on exchange resins showing the order glycine < leucine < phenylalanine < tryptophan. Cleaver observed that this was the same order in which the amino acids distributed themselves from aqueous solution into an organic phase (184), the neutral amino acids passing into the organic phase to a greater extent as the hydrocarbon portion of the molecule increased in size and aromaticity. He proposed the hypothesis that these neutral amino acids dissolved in the resin. Tests with alanine, norleucine, and phenylalanine supported this hypothesis on the whole. A further correlation appeared when he showed that the extent of the sorption of norleucine and of phenylalanine in cation exchangers followed the order of equivalent weights of the resins. It seemed, therefore, that since the resin of low equivalent weight contained more polar, hydrophilic groups per unit of mass than did the resin of high equivalent weight the latter would present more nonpolar area (or volume) for sorption of the aliphatic or aromatic nonpolar parts of the amino acids, and hence would take up per gram larger quantities of the less polar amino acids. It seemed that this phenomenon might well be encountered rather frequently with the adsorption of organic substances by these resins.

Richardson and others (228,776–778) have made analogous observations in connection with sorption and exclusion of molecules by ion exchange resins. For example, Deuel and co-workers (228) showed that the anion exchanger Amberlite IR-4B would take up low molecular weight nonelectrolyte molecules up to a certain size, but that large molecules (poly-

galacturonic acid, polymanonic acid, polymetaphosphoric acid) were not adsorbed, and so could be separated from the smaller ones. A process explicitly utilizing these behaviors was developed by Wheaton and his co-workers (see previous section, and Section X (858,989,990)).

VIII. CHEMICAL REACTIVITY

Outspoken chemical reactions may be observed when organic exchange resins are treated with oxidizing or reducing agents. These resins are usually decomposed by chlorine, bromine, and chromic acid but not by sulfuric acid, ferric iron, or, at room temperature, dilute nitric acid (47). Frizzell (302) found that Zeo-Karb could be used for a number of quantitative analytical separations, but that, at a pH of 4, 0.1 N solutions of potassium dichromate, potassium bromate, or iodate were reduced, and iodide in KI was oxidized to iodine by the resin.

IX. APPARATUS

The apparatus used in ion exchange chromatography is essentially the same as that described for column chromatography of all kinds (Chapter VI, Section III, and Chapter VIII, Section III). The only difference may lie in the methods for detecting zones. The principles presented in Chapter XII apply also to ion exchange chromatography.

X. METHODS

The following examples illustrate the use of ion exchangers in a variety of applications that are typical of their behaviors. The types of behavior illustrated are classified in Table IX-1.

1. Behavior of Clay

Clay minerals are not commonly used in ion exchange applications because of their low exchange capacities and the difficulty of working with these materials. For these reasons the only mention made here is for the purpose of leading those who are interested in adsorption on clay minerals to the literature. The problems of handling these recalcitrant substances have been solved in principle by H. C. Thomas and his co-workers (282,307,308). They have used tracer techniques to handle the small changes in concentration and large volumes of solution needed in clay work, and have supported the clay columns on asbestos as a filter aid, thus being able to prepare columns with a practical flow rate. The exchange capacity of the asbestos support was determined as 0.01 meq. or less.

2. Inorganic Separation

A. SEPARATION OF MERCURY FROM GOLD

MacNevin and Lee (587) have shown how fairly large quantities of gold and mercury may be separated. They base the separation on the differences in stability of the chloride complexes of these ions which are $K_{HgCl_4^-} = 8.3 \times 10^{-15}$ and $K_{AuCl_4^-} = 5 \times 10^{-22}$. By choosing a suitable chloride ion concentration, a maximum of auric ion and a minimum of mercuric could be converted to the corresponding complex anions, thus producing a favorable situation: mercury largely cationic; gold largely anionic; a situation analogous to that used to separate the platinum group metals (586).

Eight hundred grams of Dowex-50 is soaked in 2 N hydrochloric acid to swell it and convert it to the acid form. If this is done in a column, the acid should flow *up* through the loose exchanger, fluidizing it so that it is free to swell without shattering the tube. This is called *backwashing*. The resin is now filled into a chromatography tube. This is a tube 100 cm. long and 5.5 cm. in diameter, with a stopcock at the lower end. (In accurate small-scale quantitative work a stopcock, with its attendant grease, is undesirable.) It is convenient to fit the lower end with a standard taper female joint, and to insert in it the male joint which has a stopcock on the stem, and a perforated disk at the wide end, as in Fig. VI-2.*a*, 1 and 2. The resin rests on a pad of glass wool. The column is then washed with distilled water till the effluent reaches pH 5. The mixture containing about 400 g. gold and 8 g. mercury in 1800 ml. of 2 N hydrochloric acid was poured onto the column and the flow rate adjusted to 10 ml./min. Tests for mercury (iodide precipitation) were made on each 100 ml. of effluent. All were negative. As the last of the mixture passed into the column it was followed with distilled water until the effluent was no longer yellow (about 1500 ml. required). None of the wash liquid contained mercury. All the effluent was pooled and examined, spectroscopically by emission. No mercury was found. Total duration of the process was less than 6 hr., with only occasional attention. The mercury is displaced from the column with 2 N hydrochloric acid and the resin thus regenerated is ready for further use.

B. MICRO METHOD

This method, taken from a paper by Thompson, Harvey, Choppin, and Seaborg (924) on the chemical properties of elements 99 and 100, illustrates the precision of which ion exchange separation is capable.

Dowex 50, 200- to 400-mesh, 12% cross-linked, bead form, is graded in the hydrogen form by settling from water suspension, collecting the

fraction with a settling rate between 0.5 and 0.25 cm./min. This is the finer part of the material. It was washed alternately with 12 *M* HCl and ammonium hydroxide, and stored in the ammonium form. The developer was prepared from 0.4 *M* lactic acid (reagent grade) in conductivity water, adjusted to a pH of 4.0 to 4.5 with conductivity water saturated with ammonia, and lastly made 0.01 *M* in phenol (to inhibit mold).

The chromatography tube, 2 mm. in diameter, was enclosed in a jacket that could be heated to 87°C. by means of boiling trichloroethylene. The tapered tip of the tube protruded from the jacket. A bed of resin 5 to 6 cm. high was formed in the tube by the slurrying method, the height chosen being such as to give the desired flow rate, in this case about 2 min. for one drop of 35λ size, and then the column was heated while passing eluent through it. Air bubbles were removed by stirring the bed. Eluent was usually allowed to flow for 24 hr. so as to cleanse the bed of possible contaminating metals.

The procedure for the separation was as follows: "The flow of eluant was interrupted and the ammonium lactate solution above the resin bed was removed. The space above the resin bed was washed carefully with hot (air-free) distilled water to remove traces of lactate. Finally, two drops of water were forced through the resin bed. This procedure was then repeated with hot 0.05 *M* hydrochloric acid, and two drops of the acid were forced through the resin bed. The remainder of the acid was removed from above the resin.

"The mixture of actinide elements, dissolved in two drops of 0.05 *M* hydrochloric acid, was carefully transferred to the top of the resin bed and allowed to flow slowly through. The tube which contained the actinides was washed successively with two more drops of 0.05 *M* hydrochloric acid and then with two drops of water. These washings were allowed to wash down the space above the resin bed and to flow slowly through. The tube was finally washed with two drops of ammonium lactate solution which were transferred to the top of the column and allowed to flow slowly through the resin bed. The first drop to fall after the transfer of the ammonium lactate to the resin bed was collected as 'drop number 1'."

The space above the bed was then refilled with hot ammonium lactate, the flow rate adjusted by the height of hydrostatic head, and the analysis carried out. The results of one such analysis are shown in Fig. IX-3. Analysis was carried out by evaporating the samples of effluent on platinum plates, igniting, and measuring in suitable ways (see original papers).

3. Amino Acid Separation

Procedure of Moore and Stein (651). The original publication must be consulted for the finest details of the procedure, which are given there very exactly. The description given here is merely illustrative of what can be done.

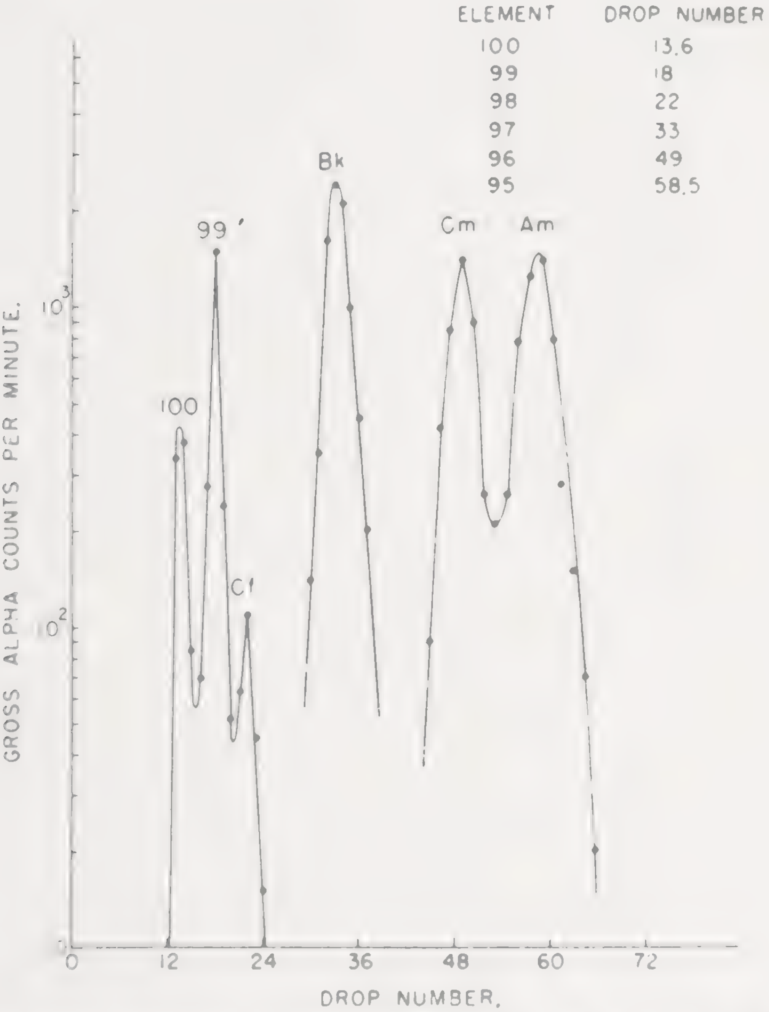


Fig. IX-3. Micro separation of elements 99 and 100, and some actinides. Elution of tripositive actinides from Dowex-50 with ammonium lactate. Drop size 35 λ; drop rate about 2 min. per drop. (From Thompson and co-workers (924).)

The chromatography tube is 0.9 cm. in diameter, and measures 165 cm. from the top to the fused-in sintered glass plate that supports the bed of resin. Below this the tube is drawn down. A stopcock is not used. The column is enclosed in a jacket through which water from a constant-

temperature bath can be circulated. The effluent is collected in a fraction collector, usually in 0.5, 1.0, or 2.0 ml. portions at a maximum rate of ml./hr. ("12 ml. per hr. per sq. cm. cross sectional area of column"). The fractions are determined photometrically with ninhydrin reagent (652).

Dowex-50, 4% cross-linking, is washed with 4 to 8 l. of 4 *N* HCl on a Büchner; twice with distilled water; then with 2 *N* sodium hydroxide until the effluent is alkaline. The resin, suspended in *ca.* 3 volumes of *N* NaOH is heated *ca.* 3 hr., with occasional shaking, on a steam bath, and let settle 1 hr.; then the supernatant is siphoned off, and replaced with fresh *N* NaOH. This sequence, heating, settling, siphoning or decanting, is repeated 4 times (total, 5 digestions) when the supernatant should be almost clear. The resin is filtered off, washed with distilled water until free from alkali, and passed through a 120-mesh sieve in 6 to 8 l. of distilled water. It is stored as the sodium salt, damp. Five pounds of commercial "through 200 mesh" resin yields enough prepared resin for "six or more 150 × 0.9 cm. columns." The resin is filled into the tube as a slurry (1:1, v/v) in the appropriate buffer, filling about 20 to 30 cm. at a time (*ca.* 50 ml. of slurry), allowing the resin to settle, sucking off supernatant, and filling some more. If the resulting column flows too slowly, the resin is removed, and suspended in liquid in a tall cylinder. The fines are then removed from the surface of the settled material and the column is repacked. It is kept under sodium hydroxide and is converted to the buffer before use. The solutions at this stage are made up to contain a detergent "to permit faster flow rates without concomitant broadening of the peaks on the effluent curves" (650) and thiodiglycol antioxidant to prevent losses of methionine (875).

The mixture, at the appropriate acid pH, *ca.* 2.5, is applied to the column (heated to the appropriate temperature) and the analysis is carried out. Figure IX-4 shows the course of an analysis of a mixture of 50 components. Amides and other non-ninhydrin-reacting substances must be analyzed appropriately. In the separation illustrated in Fig. IX-4, a gradient elution procedure was used, as shown. The method with ion exchanger "permits the recovery of most amino acids within 3 percent of theory in a single chromatogram at pH 3.1 to 5.1 by elution with sodium acetate-citrate buffers of gradually increasing pH and ionic strength" (651). For such results there must be close attention to the details of the procedures as given by Stein and Moore.

4. Sugar-Separations. Use of Borate Complexes

The use of complexing agents in inorganic ion exchange is common. It is also possible in the organic area as shown in the separations of sugars

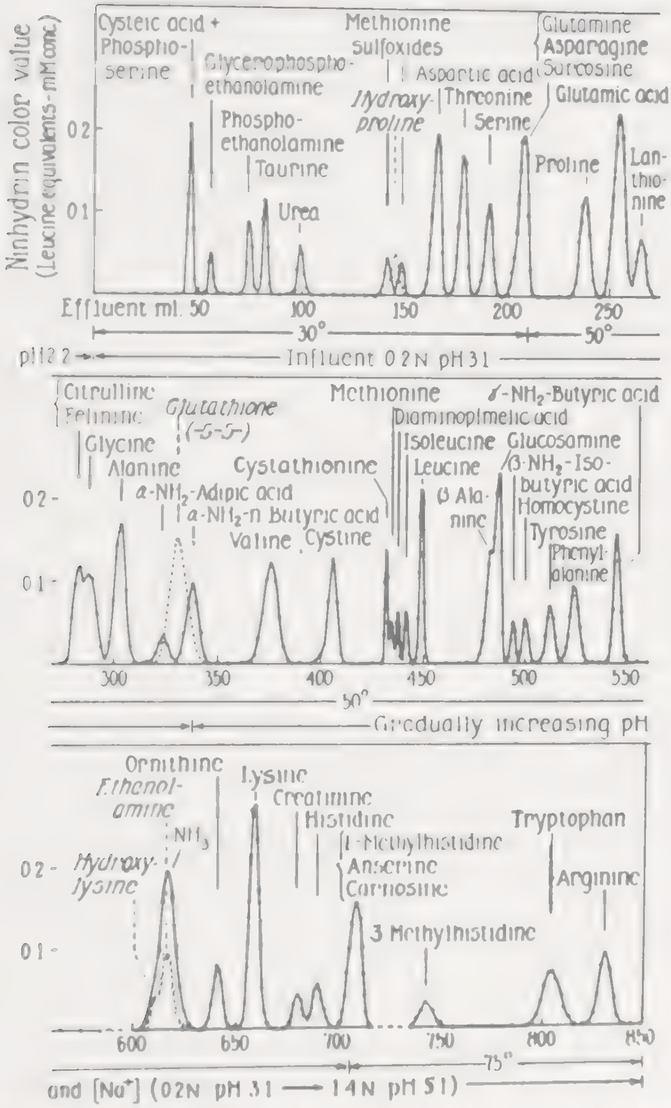


Fig. IX-4. Separation of amino acids and related compounds from a synthetic mixture containing 50 components. The column of Dowex 50-X4 (150 × 0.9 cm.) was operated with sodium acetate and citrate buffers at the temperatures indicated. The amino acids were each present in 0.05 to 0.20 mg. quantities. The effluent was collected in 2-ml. fractions except in the range from cystathionine through leucine, in which 0.5-ml. fractions were collected. The positions of hydroxyproline and glutathione, which were not included in the mixture, are indicated by dotted curves. The concentrations of hydroxylysine and ethanolamine were determined after removal of NH_3 by evaporation. The dotted base line at 600 and 725 effluent ml. indicates the positions at which there is a change in blank resulting from the elution of traces of NH_3 contained in the influent buffers. (From Moore and Stein (651).)

and their derivatives reported by Khym and co-workers (470–472,962, 1039). Borate complexes of sugars of the type sought are anions.

The strong-base anion exchanger (Dowex-1, 200 to 400 mesh) was freed from fines by settling and decantation of the slower settling material, packed by the slurry method into the column, and washed with 1 *N* HCl. The resin was converted to the tetraborate form by passage of 0.1 *M* potassium tetraborate until the chloride test in the effluent was faint, then washed with water. The column just before use was washed with dilute borate solution, then the sugar mixture in dilute borate was applied, followed usually by a little of the dilute borate solvent, then the requisite developers.

In one experiment 23 mg. incompletely hydrolyzed sucrose in 6 ml. of 0.005 *M* potassium tetraborate ("borate") was applied to a column *ca.* 1.04 cm. in diameter ("0.85 sq. cm.") $\times$ 11 cm., of 300-mesh borate form resin. It was developed with 0.005 *M* borate, when a zone of 8.0 mg. sucrose appeared, then 0.015 *M* borate, which brought out nothing, then 0.02 *M* borate which brought out 7.4 mg. fructose, then 0.03 *M* borate which brought out 7.7 mg. glucose. Flow rate was 1 ml./min. and a total of about 2.5 l. of eluent was used.

In another experiment with a column of the same size and composition running at the same flow rate, 25 mg. each of sucrose and maltose in 10 ml. of 0.005 *M* borate were applied to the column and developed with 0.005 *M* borate. All the sucrose appeared in the first 500 ml., and all the maltose in the next 800 ml., according to the figures given.

5. Ion Exclusion. Sorptive Use of Exchanger

Two sorptive uses of an ion exchanger may be pointed out: one, ion exclusion uses the exchanger to separate an ionic from a nonionic substance or substances; the other separates two nonionic substances. Both will be illustrated from data given by Wheaton and Bauman (858, 989,990). The method of ion exclusion relies on the principle (Section IV) that the concentration of mobile ionic species inside of a resin particle will be markedly different from that outside the particle, whereas the nonionic species will not show such a marked differential.

As an example (Fig. IX-5) a bed of 50- to 100-mesh Dowex-1, 10% cross-linking, strong base resin in the hydrochloride form was treated with 15 ml. of 2% sodium chloride–2% ethyl alcohol and a water wash. The two components were separated: the sodium chloride, excluded by the highly ionic resin, appeared as the first zone, and the alcohol as the second. The column was then empty and ready for a repetition of the same cycle. Analogous behavior was shown by a weak base exchanger, a strong acid, and a weak acid exchanger. The process could be made a semicontinuous one (858,990).

The resins were found to exclude some organic molecules. For example, sucrose was excluded (because of its size, perhaps) (990). It was then found that if a 62 × 1.5 cm. bed of resin containing 100 ml. of Dowex-50, acid form 8% cross-linked, 50- to 100-mesh bead size, was treated with 15 ml. of 4% glucose—4% methanol solution in water, the mixture was separated into two parts, with a zone of the larger molecular size material appearing first, and a zone of the alcohol next. This is shown in Fig. IX-6. It is a case of sorption chromatography, and might be likened to a liquid partition system: a system with a gel-like stationary phase.

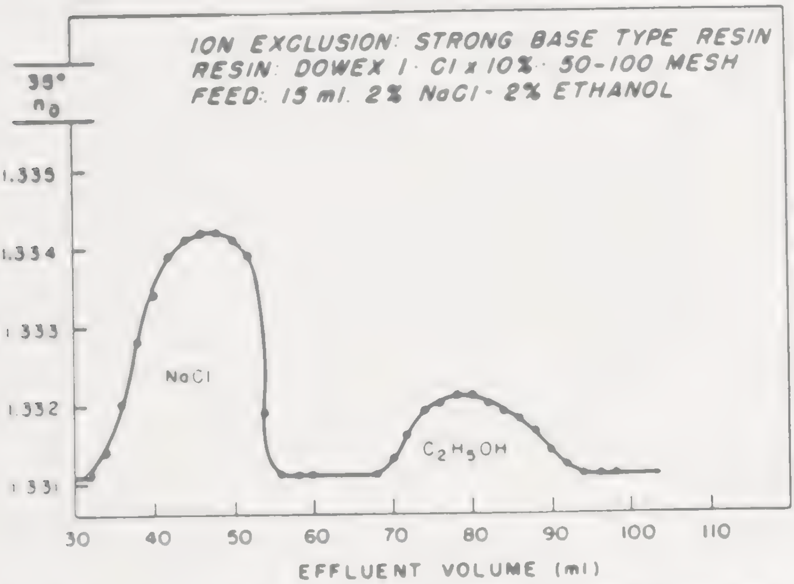


Fig. IX-5. Ion exclusion by a strong base exchanger. The column of Dowex-1, chloride form, 10% cross-linked, 100-mesh, was fed with 15 ml. of a mixture 2% in NaCl and 2% in alcohol. (From Wheaton and Bauman (989).)

Exchange resins have been suggested to act like molecular sieves (228, 776–778). Richardson (778) showed that a dyestuff of average diameter about 30 A was completely prevented from entering a resin, though there was some reaction at the surface (less than 0.1% of the available exchange centers), and this permitted purification of the dyestuff because smaller molecules were sorbed.

6. Catalysis

An example taken from Mowery (660) shows a set of catalysis experiments, with chromatographic working up of the products. One object was to determine the effect of the resin upon the distribution of isomers in methyl glucoside formation *vis à vis* that of a dissolved acid.

"The ion exchange resins used were sulfonated polystyrene resins of 1, 2, 4, 8, 12 and 16% crosslinking manufactured by the Dow Chemical Company under the trade name of Dowex 50 by incorporation of corresponding percentages of divinylbenzene before polymerization. 50–100 and 200–400 mesh sizes were investigated and for the sake of brevity these mesh sizes will be designated in this paper as coarse and fine, respectively. The resins, supplied moist in the free sulfonic acid form, were prepared for use by six 24-hour treatments with methanol followed by partial drying on a Büchner funnel. The alcohol content, determined by drying two hours at 110°, was found to be 82, 70, 57, 43, 39 and 32%,

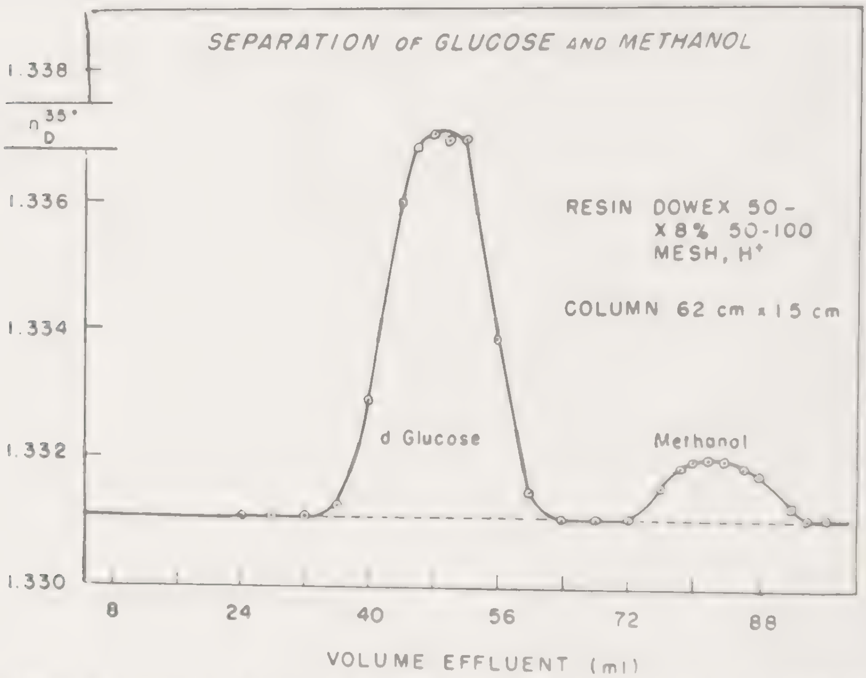


Fig. IX-6. Separation of glucose and methanol. The column of Dowex-50, acid form, 8% cross-linked, measuring 62 x 1.5 cm., and containing 100 ml. of resin was treated with 15 ml. of 4% glucose–4% methanol, followed with water. (From Wheaton and Bauman (990).)

respectively, for the 1, 2, 4, 8, 12 and 16% cross-linked materials. These resins were stated by the manufacturer to have capacities of 5.2, 5.2, 5.2, 5.1, 5.0 and 4.9 meq. per g. of dry resin, respectively. For each resin and mesh size two reactions were run, one using 4.10 g. and the other 3.85 meq. per 100 ml. of methanol.

"In a typical reaction 5.25 g. of D-galactose (Pfanstiehl C.P.) and 350 ml. of methanol were refluxed in a 500-ml. flask with good agitation. When the alcohol had reached boiling and part of the galactose had

dissolved, the resin catalyst or methanol solution of benzene-sulfonic acid was added and the time noted. One-ml. samples were withdrawn at intervals and titrated for reducing sugar (379). The reaction mixtures were worked up by simply filtering off the resin, dividing the filtrate, including the washings, into two equal parts and evaporating the alcohol under vacuum at 50°. The weight of sirup obtained, usually slightly under 3 g., was determined to the nearest hundredth of a gram. The sirup was transferred quantitatively with methanol to the top of a 4.8×122 cm. air-blown Florex XXX column (358,359). Methanol was then run through the column under about one atmosphere pressure and the column effluent, which was analyzed by passage through a 4-dcm. polarimeter tube, was separated into the positively rotating α-galactoside and negatively rotating β-galactoside fractions. These fractions were evaporated under vacuum and the weight of α- and β-isomers produced by the resin was thus determined. In some cases duplicate runs were performed and found to differ by no more than about 5% in the weights of α- and β-isomers obtained. Thus both the initial rapid formation of non-reducing methyl D-galactosides, principally methyl β-D-galactosides, and the subsequent slower formation of α- from β-isomers could be followed and compared with the corresponding reaction using dissolved benzenesulfonic acid as catalyst. Polarimetric elution curves were plotted and areas measured so that furanoside and pyranoside distributions in each of the chromatographic fractions could be calculated in the usual way (661). The benzenesulfonic acid used as catalyst in the comparison cases was removed after the reaction by passing the solution through a column of a weakly basic ion exchange resin. An anhydrous solution of benzenesulfonic acid in methanol was prepared most easily by passage of a methanol solution of anhydrous sodium benzenesulfonate through an alcohol-treated Dowex 50 column."

It was observed that with decreased cross-linking the rate of catalysis by the resin approached that of dissolved benzene sulfonic acid, and that there appeared to be a tendency for the resin catalysis to lead to a slightly higher yield of α furanoside than the free acid produced.

7. Other Applications

Other applications of ion exchange resins, though not chromatographic, may be referred to by enumeration by way of indicating the breadth of usefulness of these substances (695a). Wherever possible, reviews are referred to. Membranes have been prepared (181,867); ion exchanger loaded with antigen has been used to separate antibody (436); the theory of ion exchanger electrodes has been developed (814); unsuccessful use of a polymer made from optically active monomer to resolve racemic mixtures has been reported (141).

Electron Exchange Polymers (Redox Resins or Polymers)

I. PRINCIPLE

Electron exchange polymers bear partial analogy to cation exchange polymers in that whereas some of the latter will exchange protons for other cations present in the ambient medium, electron exchangers will exchange electrons (166). The analogies that connect acid-base with oxidation-reduction chemistry (179,632,830,838) also connect ion exchange chemistry with electron exchange chemistry. The analogies extend to the chromatographic application of these substances. Whereas a column of cation exchanger in the acid form will release protons if treated with an exchangeable cation, a column of redox resin in the reduced form will release electrons (and an equivalent amount of protons) when treated with a suitable oxidizing agent. In both cases, formally analogous breakthrough curves are obtained at saturation of the column.

II. CLASSIFICATION

Redox polymers can be divided into two groups: one in which the only electrolyte groups are the redox groups; and one in which both redox and ion exchange groups are present in the same polymer. The latter can be considered the most general case of a chemically active polymer, namely, a combined redox and acid-base polymer. Ion exchange resins are a special case of this type of polymer, and redox polymers are a special case wherein although ion exchange properties may be present in the reduced and/or oxidized forms, they are *not* independent of the oxidation state of the polymer.

Redox polymers can be classified into groups in terms of whether the functional group is reversibly oxidizable or not. The former case alone will be considered in this chapter. They may be classified in terms of whether the functional group is part of the backbone of the chain (class 1) or whether it is a substituent on the chain (class 2) (166). In the former case the shift from reduced to oxidized form or the reverse might be expected to have effects on chain length and on mid-point potential different from those observed with substances belonging to class 2. A further classification then follows depending on whether the redox

function is oxygen–carbon only, as in polyvinyl hydroquinone or polyvinyl catechol groups (166,504,600–602,868,952,961); oxygen–carbon and nitrogen–carbon, as in 2-hydroxy-5- aminostyrene polymers (278); nitrogen–carbon as in butyl polyvinyl pyridinium bromide (166) and related substances; sulfur only as with polyvinyl thiol (166), or polythiol-styrene (347,706), or *N*-mercaptomethylpolyamides (152) or one of a number of practicable inorganic atoms. The polymers and copolymers of dicyclopentadienyliron (17,980) are another class of electron exchanger as are the copper- or silver-bearing amine ion exchange resins of Mills and Dickinson (644) (see footnote to Table IX-4) and the cation exchangers loaded with metal (ferric/ferrous; stannic/stannous) or anion exchangers loaded with organic molecules, as methylene blue/leucomethylene blue or quinone/hydroquinone reported by Sansoni (808). It will be noted that these different types may be classed in terms of the way that hydrogen ion enters the redox reaction, or, perhaps more clearly, they may be divided in terms of whether the oxidized form (*a*) carries no charge, as in the case of benzoquinone or a disulfide or (*b*) carries a positive charge, as would be the case of a butyl polyvinylpyridinium halide (166).

From a practical point of view the electron exchangers can be divided into classes depending on whether the polymer, either with reference to the chain or the attachment of the functional group, is stable, or labile, to acid and/or base. The polyvinyl hydroquinones (166,952) represent members of the first class; in the latter class are the polyamides (152,166) or the polyacetal types (240,589). Further classification might be based on whether the polymer is a linear or a cross-linked one, the classification being applicable to all the above types (166,171,173,277,279). Polymers have been prepared, also, which incorporate a naturally occurring redox group into a polymeric substrate (526,527), and these may be placed in the appropriate class above.

The term electron exchange was used by W. M. Clark in connection with a discussion of valence change, and, in general, redox phenomena (180). It was applied to these polymers in the first paper on this subject (166).

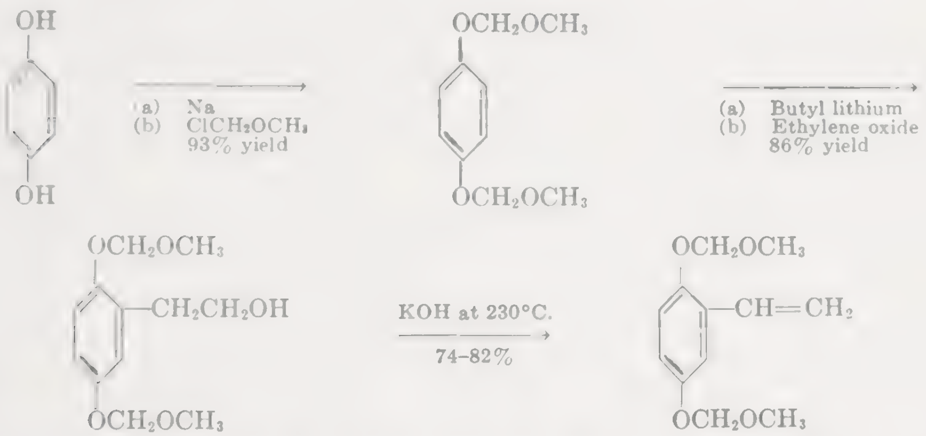
III. PREPARATION

The preparation of these substances can be found in many of the cited references. Examples are given of two types upon which most of the published work relevant to this chapter has been done.

1. Vinyl Hydroquinone Polymers

Vinyl hydroquinone polymers can be prepared from the monomers vinyl hydroquinone diacetate (279,774), dibenzoate (279), or bismethoxy-

methyl ether (880). These monomers may be copolymerized in the conventional ways with other vinyl monomers. An improved and very generally applicable scheme for introducing a vinyl group into a phenolic ring is as follows (880):



Metallic sodium, 13.8 g. (0.6 g. atom), was reacted with 200 ml. of absolute methanol. The solution was stirred while 66.0 g. (0.6 mole) of hydroquinone in 200 ml. absolute methanol was rapidly introduced, the temperature of the deep orange solution being maintained at 0°C. by external cooling. A small excess over the theoretical 48.0 g. (0.6 mole) of chloromethyl methyl ether was added over a period of 30 min., until the solution reached a pH of 8.

This sequence, from the dissolution of metallic sodium through the introduction of chloromethyl methyl ether was repeated thrice, each time with amounts equivalent to 0.6 mole, so that a total of 54.2 g. sodium (2.4 g. atoms) and 192.1 g. chloromethyl methyl ether (2.4 moles) were used. After stirring at room temperature overnight, the mixture was filtered from precipitated sodium chloride and the solvent evaporated. The residue, taken up in ether and purified by extraction with sodium hydroxide, yielded 110.0 g. of crude hydroquinone bismethoxymethyl ether. Fractional distillation yielded a colorless liquid, b.p. 75°C. at 0.3 mm., $N_D^{25.0}$ 1.4972.

A standard solution of n-butyl lithium was prepared from 70 g. (10 moles) lithium and 370.4 g. (4 moles) *n*-butyl chloride in 1200 ml. dry ether. The resulting solution was found to be 1.67 *N* in *n*-butyl lithium.

To a solution of 89.2 g. (0.45 mole) of hydroquinone bismethoxymethyl ether in 75 ml. dry ether was slowly added 298 ml. (0.50 mole) of 1.67 *N* n-butyl lithium solution at room temperature. A white precipitate soon appeared, and the mixture was stirred for 24 hr. The mixture was cooled at 0°C. and held at this temperature while liquid ethylene oxide, 26.4 g. (0.6

mole) was distilled *very* slowly into the reaction flask, which was protected by a dry ice condenser. After being stirred overnight at room temperature the reaction mixture was poured into 500 ml. ice water. The water was extracted with ether, the organic layer dried, and the solvent evaporated. Fractional distillation yielded 21.8 g. starting material and 71.4 g. of crude β-(2,5-dihydroxyphenyl)-ethanol bismethoxymethyl ether, boiling at 148° to 156°C. at 0.4 mm. Fractional distillation yielded colorless liquid, b.p. 173°C. at 2 mm. $N_D^{20.0}$ 1.5149.

To dehydrate the alcohol, a 300-ml. two-neck flask was fitted with an addition funnel and a small distilling head. Into it were placed 70 g. U.S.P. potassium hydroxide pellets and 1 g. picric acid as polymerization inhibitor. The apparatus was evacuated to 5 mm. and heated to 230°C. The potassium hydroxide melted, and a rather violent evolution of gas ensued. After this had ceased the hydroxide in the flask was solid. The alcohol, 36.4 g. (0.15 mole), was now introduced at a rate of about 10 drops per minute. A distillate came over at 135° to 158°C. and weighed 33 g. Distillation in the presence of a trace of picric acid yielded a colorless liquid, b.p. 107°C. at 0.1 mm. $N_D^{23.0}$ 1.5263. This material, the bismethoxymethyl ether of hydroquinone, could be hydrolyzed to vinyl hydroquinone by methanolic hydrochloric acid.

With the monomer available polymers and copolymers can be prepared by conventional means (346). Oxygen must be carefully excluded. No conditions that will cleave the protecting groups can be used. This is important in the case of radical polymerization since free quinone is an inhibitor. The preparation of cross-linked redox bead polymers, analogous to ion exchange bead polymers, is carried out by suspension polymerization (278,279), followed by a sulfonation step that produces water-wettable and swellable material.

Linear polymer is readily prepared (278). For example, 2,5-dibenzoxystyrene and α-methylstyrene 1:1 molar ratio are dissolved in enough toluene to give a 40% solution. About 0.1 mole % benzoyl peroxide or other initiator is added, and the mixture is heated at about 58°C. for $4\frac{1}{2}$ days, followed by increased temperature, 70° to 90°C. for several more days. A viscous solution results. This is diluted with toluene, and precipitated by addition to methanol with vigorous agitation. Dissolution in toluene and reprecipitation, repeated five times, gets rid of low molecular weight material. The purified polymer is dissolved in benzene and freeze-dried. This polymer is not soluble in water, but dissolves in benzene or toluene and in glacial acetic acid or tertiary butyl alcohol.

The polymer can be made water soluble by sulfonation. Forty milligrams are dispersed in 1 ml. concentrated sulfuric acid at 0° to 5°C. for 35 to 40 min., or until a drop of the liquid dissolves completely in water

The solution is then diluted by pouring it into water, and the sulfuric acid removed by dialysis. In the process of sulfonation the benzoyl protecting groups are cleaved, so that the polymer must be protected from oxidation.

Cross-linked bead polymer can be prepared as follows (278). Vinyl hydroquinone dibenzoate (5.00 g.) and α-methyl styrene (1.737 g.) (1:1 molar ratio) were mixed with 0.712 g. commercial divinyl benzene mixture. Benzoyl peroxide, 0.111 g., was added. The mixture was warmed gently and added to a stirred mixture of (62 ml.) water and (1.8 g.) soluble starch, heated to 80°C. Beads of a suitable size (0.5 to 1 mm.) were produced by adjusting the rate of stirring. These hardened in $2^3/_4$ hr., and after 6 hr. the stirring was stopped. Heating was continued for 4 more hours. The beads were filtered from the cooled solution, washed thoroughly with cold water to remove starch, and then extracted in a soxhlet with benzene or toluene to remove monomers and low molecular weight material. At this point the beads were swollen and translucent.

The polymer was sulfonated as follows (991). Dried beads, 0.2058 g., were swollen with hot benzene in a sintered glass funnel attached to a suction flask. The excess benzene was removed. The beads were then treated with 2 to 3 ml. conc. sulfuric acid for $2^3/_4$ hr., becoming reddish brown. The acid was sucked off, and the beads were treated successively for several hours or overnight each time with 90%, 80%, 60%, 40%, 20%, 10%, and 5% sulfuric acid and finally water. The beads were at this stage readily wetted by water, nearly colorless, and highly swollen. It is difficult to remove traces of residual acid from them.

2. Hydroquinone Formaldehyde Polymers

Hydroquinone formaldehyde polymers (166,600–602,961) have been prepared and studied by Manecke. He found that it was desirable to copolymerize phenol or resorcinol with the hydroquinone and formaldehyde to improve the stability of the polymer. A ratio of hydroquinone, phenol, and formaldehyde, 1:1:2.5, was used to prepare material for break-through studies (602).

Many different procedures may be employed to polymerize these substances (158) (Manecke gives no explicit directions). One procedure is as follows. The hydroquinone, phenol, and formaldehyde are mixed and blown gently with carbon dioxide or nitrogen to remove dissolved oxygen. The catalyst, hydrochloric acid, is added in an amount necessary to bring the final concentration to from 0.4 to 2 *N*. The mixture is then refluxed until the polymer is formed and separates. Tan-colored resin is thus formed. On heating at about 180°C. it yields a hard, infusible polymer. This is ground and sieved before use.

IV. BREAK-THROUGH BEHAVIOR

An example of break-through behavior is as follows (573). Air-dry beads, 0.96 g., form a bed 4 cm. deep in a 50-ml. burette. Passage through this bed of 5% KI in 3 N sulfuric acid yields iodine equivalent to 1% of the calculated redox groups, indicating slight oxidation during preparation and bringing the bed into the reduced form. A wash with 3 N sulfuric acid removed the iodide and brought all the sulfonic groups into the acid form. The excess acid was washed out of the bed (until the effluent was no longer acid) and then the bed was treated with 2 M KCl. The K^+ ions, in high concentration, replaced protons of the sulfonic acid groups, and the effluent became acid. The total acid released by the column was titrated. The column was then washed with sulfuric, with water, and again treated with 2 M KCl. A reproducible cation exchange capacity of 3.89 meq./g. on a vacuum dry basis was found. All solutions were deaerated before use. The exchange was quite rapid, and fairly sharp break-through curves were obtained.

The column could slowly reduce and more rapidly oxidize a solution. When treated with bromine in 3 N sulfuric, the bed showed a total capacity of 5.70 meq./g. on a vacuum dry basis; reduced with 5% KI in 3 N sulfuric after washing out all oxidizing agent, the bed capacity was 5.63 meq./g. Again oxidized with bromine in 3 N sulfuric after washing out all reducing agent, the capacity found was 5.54. Washed and again reduced the observed capacity was 5.71. Washed and reoxidized with bromine in pH 4.7 1 M acetate buffer the capacity was 5.56 meq./g.; all were calculated on a vacuum dry basis.

Break-through curves were obtained with columns of hydroquinone–formaldehyde–phenol or resorcinol resins by Manecke (601), who found the values 7.19 and 7.06 meq./g. for two oxidations of a 1:1:2 hydroquinone: resorcinol: formaldehyde resin, and 6.67 and 6.57 meq./g. for a 1:1:3 resin (602). He has discussed the shape of the break-through curves as it is affected by the potential difference between that of the exchanger and the oxidant: the curve will be steeper the greater this difference. The curve is also steeper the slower the throughput of solution and the smaller the particle size of the resin. These observations held for the reduction cycle also. The capacity of the bed was increased by slowing down the flow rate; this suggested that diffusion into the particles played a role, but the capacity was somewhat lowered with smaller particles. Increased temperature (from 20° to 45°C.) did not affect the shape of the break-through curve but doubled the capacity of the bed for oxidation by ferricion. The concentration of the oxidant affected the break-through capacity, and it was indicated that optimum values of concentration and throughput must exist that will show the maximum capacity of the

bed. The author also points out that it is expected that these columns should behave in a manner analogous to ion exchangers.

V. RADIAL-FLOW APPLICATION

Water-insoluble linear polymer (unsulfonated) was dissolved in a small amount of glacial acetic acid and used to impregnate pieces of filter paper. The filter paper, dried and freed from acid by evacuation over KOH in a desiccator, was placed between glass plates, as illustrated in Fig. VII-4. The upper plate had a *ca.* $^1/_{16}$-in. hole in the center. If the polymer is in the reduced state, a drop of iodine in potassium iodide in pH 7 phosphate buffer, introduced to the paper and washed in with buffer, first forms a dark zone, which then is decolorized. A starch test on the paper shows no iodine. If the starch test is not made, but the iodide is washed from the zone with buffer, and then a drop of acid KI is introduced, iodine immediately appears (starch test), indicating that a zone of oxidized insoluble material was present. Such impregnated papers can be oxidized, if in the hydroquinone form, by ferric ion, and presumably many oxidizing and reducing agents may be put to test with them.

VI. POISING AGENT USE

Ion exchangers can be used as buffering agents. Electron exchangers can also presumably be used as poising agents. An example of this using a coated ion exchanger has been provided by Malin and Finn (598). These investigators used an oxygen-absorbing resin, Duolite S-10 (644), in the reduced form, to maintain anaerobic conditions in a bacteriological medium. The resin worked well under conditions where a molecularly dispersed reducing agent, thioglycolate, gave poor results.

VII. CONCLUDING COMMENTS

This chapter is included in this volume chiefly because recent reviews of ion exchange chemistry have called attention to the redox polymers and thus pointed an analogy to ion exchangers (432,511a,829).

The analogies are undoubtedly even closer, in terms of operating phenomena, than have been indicated here. Their elucidation waits on progress in the study of these substances.

Foam and Emulsion Fractionation

I. PRINCIPLE

The separation depends on the distribution of the substances between a bulk liquid phase and the interfacial film between this phase and bubbles or droplets formed in it. The bubbles or droplets move through the bulk liquid which is stirred by their passage. This is a chromatographic process (164). The interfacial phase moves against the bulk phase in a differential countercurrent manner. The more surface-active components of the mixture distribute to a greater extent into the interfacial film, which may be stabilized by their presence (though the question of what factors control foam stability seems still to be an open one). By drawing off the foam or emulsion the molecular distribution which enriched it may be converted into a bulk separation of the components of the original mixture (Chapter I). The earliest systematic work in this field goes back at least to 1900 (3).

II. CLASSIFICATION

In foam fractionation the distribution is of the L-M type, where the "mobile" interface (Chapters I and II) is formed between liquid and vapor. In emulsion fractionating, the mobile interface lies between two liquids. Here an additional distribution process may occur, for solute may pass from the bulk liquid not only into the interfacial film but also into the (bulk) liquid inside the droplets of the emulsion. This disturbing effect may not be important, and is of course possible also in the foam-fractionation case if the adsorptive is volatile.

In foam fractionation the fractions are collected from the surface of the foamed liquid, and then are allowed to break, or are broken by the addition of antifoam agents, or by drying in a desiccator. In emulsion fractionation, if the droplets are lighter than the main body of fluid, the emulsion creams and the cream fraction can then be separated. If heavier, the emulsion is collected through the bottom of the vessel. In this chapter primary emphasis is placed on foam separations.

Both foam and emulsion chromatography differ in a trivial respect from ordinary chromatography in that the interface is produced in the

solution by the formation of bubbles or drops, whereas in chromatography the mobile phase replaces another interfacial phase when it contacts the stationary phase. These fractionation procedures are the molecular-level analogs of flotation. Since the term "flotation" applies to micro- or macroscopic particles, it should not be used in connection with molecular particles.

Foam and emulsion fractionations may be used to remove a substance from a mixture, possibly concentrating it and separating it from other components, or they may be used to remove impurities and leave the desired substance behind. A distinction has to be made in this connection between concentrating a material in a foam and enriching it (700). The total solids in a mixture may be *concentrated* in a foam or emulsion (through adsorption to the interface). At the same time if one component is *preferentially* taken up, the foam will be *enriched* in it in relation to the original mixture.

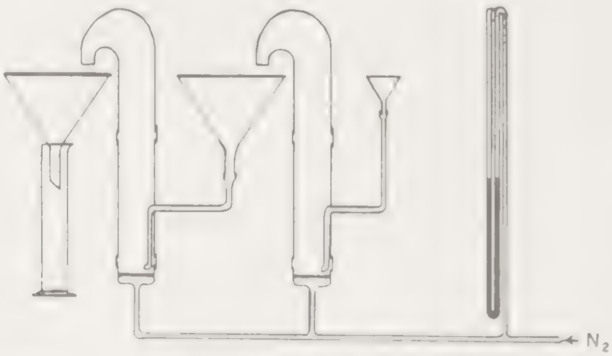


Fig. XI-1. A cascade foaming device. The material to be foamed is introduced through the small funnel onto the porous plate. The foam produced from this is collected and refoamed in a two-stage process. (From Ostwald and Mischke (699).)

Both foam and emulsion fractionations can be carried out by batchwise or by cascade, or by countercurrent procedures. The cascade is, as stated earlier (Chapter I), a systematic repeated batchwise process. (See Fig. XI-1.)

In the *batchwise process* a given volume of liquid is shaken in a vessel to produce a foam. The shaking is stopped, and the foam layer allowed to gather—a process during which bubbles rise through the liquid to the surface, and excess liquid entrained by the foam drains back. Alternatively, the foam layer may be produced by bubbling a certain amount of gas through the liquid and then stopping, and allowing the foam to gather. In both cases such a foam is called a *static foam*, since it is formed and then not replenished. It may be very *stable* and last a long time or

it may be *unstable* and *break*, decreasing in volume and returning the materials constituting the liquid part of it back to the solution. The batchwise bubbling process has in it some elements of frontal analysis, but as there is drainage of fluid from between the bubbles back to the liquid, the analogy is not exact.

The *countercurrent process* consists in bubbling a gas through the liquid and building up a foam. This foam may be so stable as to rise in the vessel in which it is formed, until all the material that stabilizes it is exhausted from the residual liquid in the vessel, when no more foam is produced. In other cases, the foam may rise to a certain height, where the level of the top layer may remain more or less steady as bubbling is continued at a constant rate. These foams produced by continuous bubbling are termed *dynamic foams*. When the top level remains essentially at a constant height (allowing for some fluctuation) and shows no tendency to rise or fall continuously, and when the liquid level below the foam has reached a constant level, the foam is said to be in a *steady state*. In such a foam, destruction of bubbles keeps pace with supply. Liquid drains down through such a foam, as the films between bubbles become thinner and as liquid is produced by collapsing bubbles. This produces a "reflux," which, especially in the early stages before the steady state is reached, has some aspects of development.

The foam fraction, when it has broken, yields a *spumate* (700). London and co-workers (565) call the residual liquid a "frothate," but this term will be avoided here, and the more common terms *residue* or *residual liquid* will be used.

III. APPARATUS

1. Batchwise Processes

For batchwise processes, the apparatus needed can be quite simple. Schütz (833) uses a 50 ml. glass-stoppered measuring cylinder, in which the liquid is made up to the 30.0-ml. mark. The cylinder containing liquid is shaken at two shakes per second, with an amplitude of about 30 in. for 15 sec. This preliminary shaking functions to saturate the atmosphere in the cylinder. It is then put in a water bath, stirred, by rolling, for 15 min., removed into a towel and shaken as above 10 sec., and returned gently, so as not to jar the foam, to the water bath for observation. The time from the stopping of shaking to the moment the center of the liquid becomes free from foam is defined as the *foam time*, T_f.

Nakayuki (677) used 20 ml. of liquid in a 40-ml. glass-stoppered test tube 1.8 cm. in diameter, shaken up and down 3 times per second with an amplitude of 25 cm. for 30 sec. He defined *foaminess* as the maximum

height of foam produced, A_m (cm.), and *foam strength*, τ (sec./cm.) as the reciprocal of the collapsing velocity of the foam. When the foam lasts longer than 30 sec., $\tau = 30/(A_m - A_{30})$, where A_{30} is the height of the foam 30 sec. after the instant of maximum foam height. When the foam does not last 30 sec., $\tau = t/A_m$, where t is the duration in seconds of the foam measured until some liquid surface free from foam appears (Schütz' T_f). He then defines *foaming ability* F as the product of τ and A_m. There are many approaches to measuring foam stability and other properties of foams, emulsions, droplets, and surface films, but a discussion of this science does not seem to belong here (73,74,195,230,368,777,1023).

2. Countercurrent Processes

In common with other chromatographic procedures, countercurrent processes can require quite simple apparatus. (Emulsion separations will not be explicitly referred to in this section, but their requirements are very similar to those for foam separations, with simple and obvious extensions from gas to liquid "developer" phases.)

What is needed is a vessel to contain the liquid to be foamed, a source of steady and controllable gas, a device for producing bubbles of suitable sizes, and a device for collecting the foam (20,341,720).

Examples of apparatus for foam separations are illustrated in Fig. XI-2. The vessel or chamber to contain the liquid to be foamed may consist of the lower part of a straight tube (g). It may be a bulb (h), or a suction flask (g) above which is fastened the foam-collecting tube. In the latter cases, the walls from the chamber to the foam-collecting tube should be tapered, so as to avoid shoulders that might trap foam. London and coworkers (565) used vessels of type (g) for foaming labile substances. They used a low depth of liquid in the chamber because they observed that increased denaturation of certain enzymes occurred when the gas bubbles passed through a deep layer of solution. However, in general, a deep layer of solution is necessary to good procedure (see below).

Various types of orifice are used to produce bubbles. Schütz (382) uses a single fine hole (Fig. XI-3,A) or a set of uniform holes (23,834). A convenient multijet constructed from short lengths of capillary tubing is illustrated by Oswald and Siehr (700) (Fig. XI-2,b). Miles (636) has pointed to one advantage of a single-hole orifice, or an orifice with uniform holes, namely, that a foam of uniform bubble size may be obtained. With such a foam some notion of interfacial area is possible. He calculated that 1100 ml. of a foam containing bubbles of 0.017 ml. volume enclosed an interfacial area of about 2 × 10⁴ sq. cm. This is enough area to accommodate several milligrams of closepacked higher fatty acid

molecules. Abribat (2,3) used a porous cup or thimble (such as is used in some ultra-filters) (Fig. XI-2,*c*). Miles, Shedlovsky, and Ross (636, 638) used platinum or stainless steel spinnerettes, commercially available from platinum fabricators and laboratory supply houses (Fig. XI-2,*d*). For his experiments on the control of foaming, Pattle (716) used Schott glass culture vessels, about 42 sq. cm. in cross section, fitted at the bottom with a sintered glass disk of pore size 100 to 200 μ (Fig. XI-2,*e*). London and co-worker (565) used Corning sintered glass disks and cylinders of different grades ("extra coarse," 160 μ average pore size; "coarse," 40 μ;

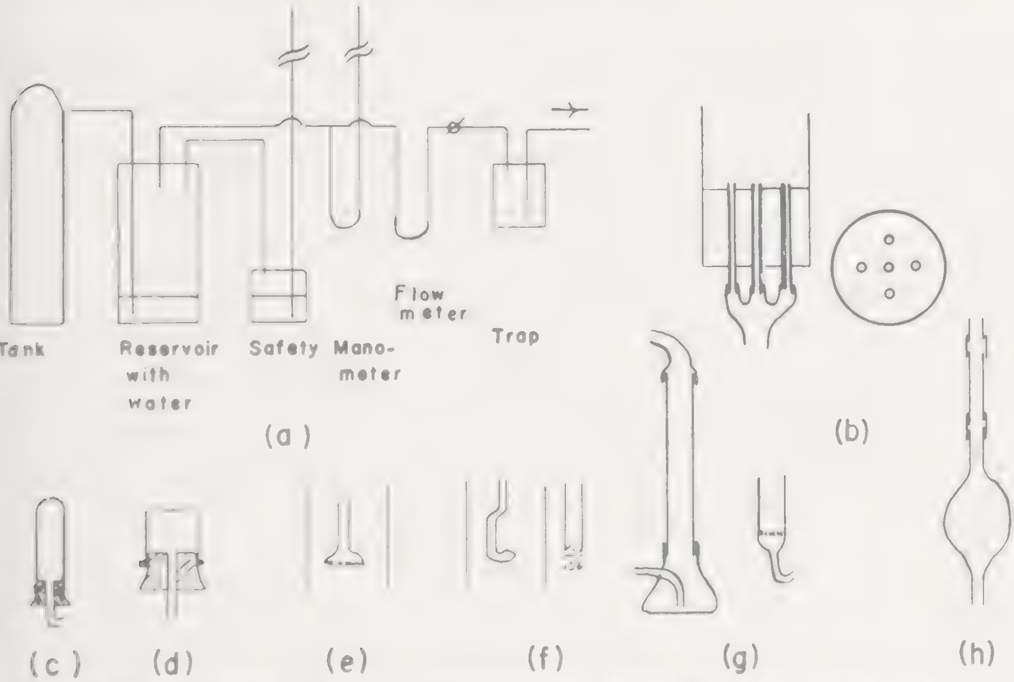


Fig. XI-2. Apparatus for foam fractionation. (*a*) The type of train used by London and co-workers (565). (*b*) Multiple-jet orifice (700). (*c*) Alundum thimble. (*d*) Spinnerette (636). (*e*, *f*) Sintered glass gas dispensers. (*g*, *h*) Vessels of various shapes (2,3,565).

and "medium," 14 μ) as well as a stainless steel dispersion tube ("D," 65 μ) from "Scientific Glass," and a spinnerette containing five 0.05 mm. diameter holes.

The removal of foam samples may be brought about in several ways, apart from reaching into the apparatus and sucking out the foam. Abribat (2,3) gained access to the column of foam by using as a container a tube made up of cylindrical segments fastened with sleeves or fitted with standard taper joints. This allowed the entire tube to be dismantled. For small volumes of foam he used a thin-walled tube that could be cut into

pieces to give access to the foam. Both he and Schütz (23,834) also used tubes designed so that by control of the gas rate the foam could be carried over, out of the tube into collecting vessels (Fig. XI-3).

The *gas* used in producing bubbles should be available in a steady stream. London and co-workers (565) found it necessary to bubble their gas through water in order to avoid "clogging" in the disperser tube. They found, in the foaming of urease and catalase preparations from buffered solutions, that carbon dioxide was better than nitrogen.

Peters (723) has developed a device for comparative study of static and dynamic foams. It consists of 30 indentical tubes arranged side by side

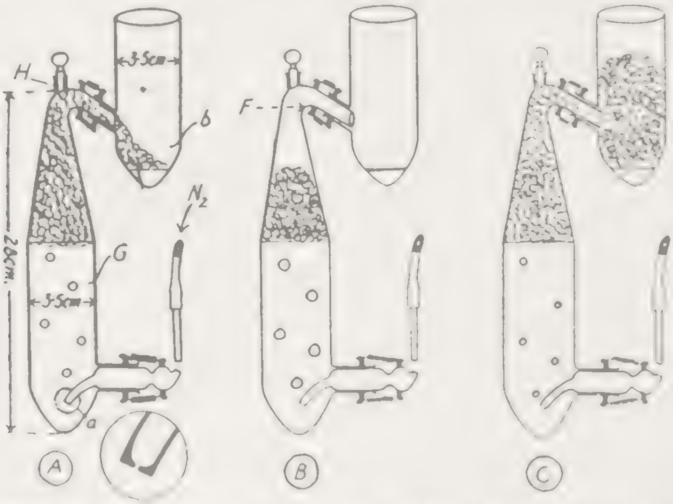


Fig. XI-3. Apparatus for foaming a solution. (*A*) A foam of medium stability is shown. Many of the bubbles break before they reach the side vessel. (*B*) When a component has been removed there may be a sudden decrease in foam stability. (*C*) Sometimes there is a sudden increase in foam stability (see text). (From Schütz (834).)

in a glass-walled thermostat. A set of 30 canulas projecting from a manifold can be lowered simultaneously, one into each tube, and bubbled simultaneously. The arrangement permits the ready comparison of the effects of a gamut of concentrations.

IV. PROCEDURES

Very complete description of foaming procedures has been given by Abribat (3) and Schütz (832,834), and this section relies chiefly on their reports, though supplemented from other sources. (74,700,723,847)

Apparatus used by Schütz is shown in Fig. XI-3. It consists of a foaming chamber and detachable receiver. The gas is introduced through a

capillary which is interchangeable, and the liquid to be foamed can be introduced through a glass-stoppered opening at the top. The apparatus is thermostatted.

In operating the apparatus (or any others, such as those shown in Fig. XI-2) the object is to form bubbles in the liquid and allow them to rise through a sufficient height of liquid that equilibrium between solute in liquid and solute at the surface of the bubble is closely approached. Very probably, equilibrium is not actually reached in any of these dynamic systems. Schütz found in his work that good results ensued when the liquid height was as shown in the figure.

Generally, the gas flow is started before liquid is introduced, so as to avoid getting liquid into the capillary. As the foam collects, entrained liquid runs off from between the bubbles and trickles back through the foam. This foam drainage functions as reflux, and as the liquid flows down through the accumulating foam, continued opportunity is given for the more surface-active molecules in it to replace the less active ones in the bubble films. The gas flow is adjusted so that most of the bubbles just reach the top of the vessel, where the neck bends, before bursting. Some pass over into the receiver. The majority burst and contribute to the drainage fluid a liquid enriched in the most surface-active components. Total "reflux" would occur if no bubbles passed over into the receiver; the case described corresponds to partial reflux.

With the apparatus functioning in this way, a sudden change in foam stability becomes immediately noticeable, as shown in Fig. XI-3,B, because the foam stops coming over and falls back in the chamber. This occurs when the component producing the foam is exhausted. At this point, the receiver is changed and the next fraction is collected by increasing the gas pressure, which increases the rate of bubble formation. The pressure is again adjusted so that most bubbles burst and contribute to reflux while some pass on into the receiver.

Aribat (3) describes a procedure that is slightly different, but demonstrates the same principles. The solution to be foamed is contained in a long tube (Fig. XI-4). At the bottom of the tube is a porous thimble or other distributor for gas. (According to Schütz, the control of whose method relies on observation of changes in foam stability, it is undesirable to use distributors such as disks or thimbles of sintered glass or alundum that yield bubbles of various sizes, since changes in foam stability are thereby masked. Further, very slow foaming is difficult with such distributors.) The liquid to be treated is introduced into the apparatus, and the height of the liquid is marked as an origin (o).

When the gas is introduced, bubbles form and rise to the surface of the solution, accumulating as a foam. The bubbles carry entrained liquid.

and thus as the foam builds up, the level of the liquid decreases from (o) (Fig. XI-4.I). Eventually, the level of the liquid falls to a maximum extent (h) (Fig. XI-4.II), at which point the volume of return of drainage liquid just balances the volume of liquid being carried into the foam by entrainment. Thereafter, the level of the liquid gradually returns close to the original value (o) (Fig. XI-5). At the same time, the height

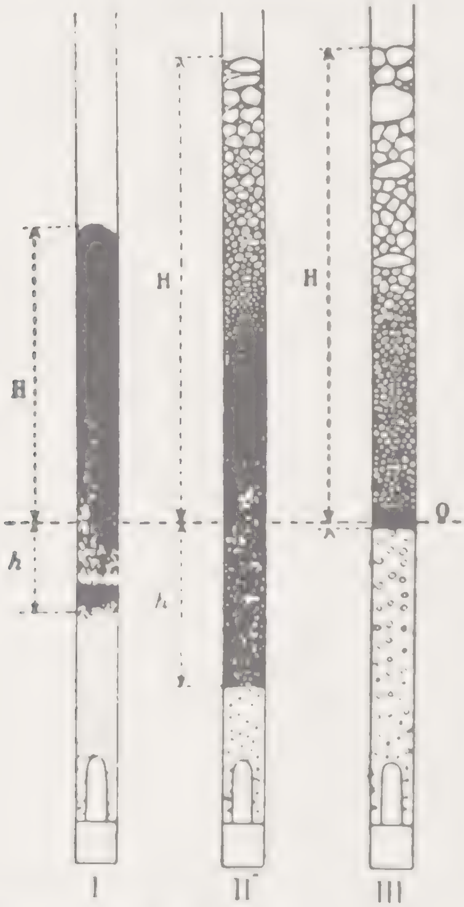


Fig. XI-4. Stages in foaming. I. The foaming has begun and wet foam is forming. II. The liquid level has fallen to a minimum, owing to entrainment in the foam. III. The foam has reached a steady state, and has drained so that the liquid level has returned close to its original value and has taken a steady value. [From [illegible]]

of the foam (H) (measured from (o)) increases to a maximum value (H) (Fig. XI-4.III), where the upper level fluctuates slightly as large bubbles break (Fig. XI-5). At this point the rate of volume contribution of new bubbles to the foam, the volume loss due to bubbles breaking, and the volume difference between drainage and entrained liquid are in balance. From this point on, the height of the foam column (H) remains

essentially constant while drainage continues until the steady state is reached (Fig. XI-4.III). For suitable fractionation the tube must be sufficiently large to contain all the foam up to the steady state, and also there must be enough residual liquid present at all times so that gas is never blown directly into the body of foam. In practice, the liquid level at point (h) should never fall below about one tube-diameter above the gas disperser.

During the period up to the steady state, the drainage liquid from the foam plays the role of "reflux" as described above. When the steady state

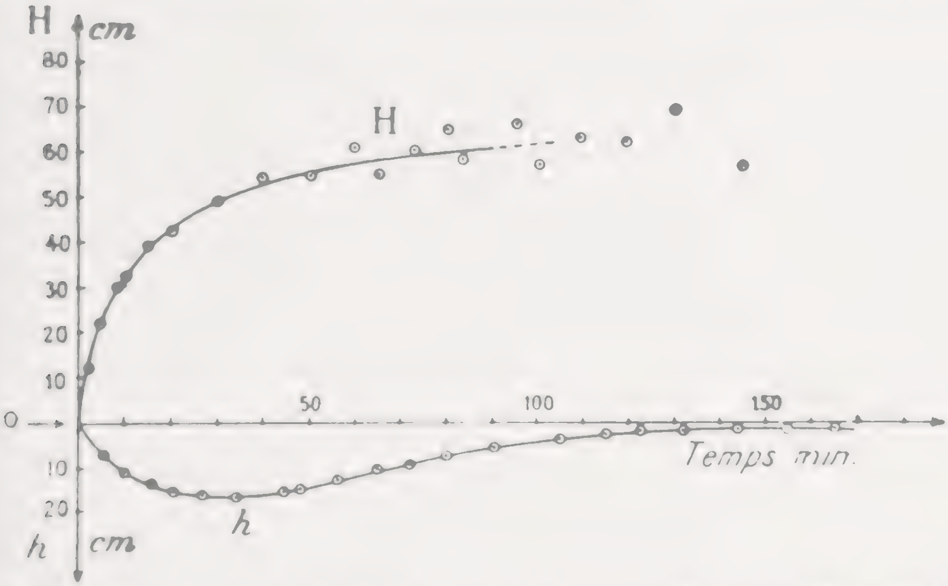


Fig. XI-5. The height of the foam, H, and the lowering of the level of the liquid, h, are plotted against the time. The foam height becomes essentially constant (within some fluctuation) as the steady state is reached. The liquid level first falls as the foam builds up, carrying entrained liquid; then as the foam drains, the liquid height returns near to its initial value. (From Abribat (3).)

has been reached, the foam is then as "dried out" and concentrated as it will be, and without stopping the flow of gas, it may be removed from the apparatus. It may even be found to contain solid material, as described below, and may not only be concentrated in one component of the mixture but also enriched.

The procedure may be continued without removing the column of foam if at the time the steady state is reached, the pressure of entering gas is increased. Then the entire sequence is observed again. The greater rapidity of bubbling allows less stable bubbles to accumulate in the foam below the more stable upper zone of foam first formed, so that a new zone builds up, eventually reaching a steady state, when the process may be repeated by

again increasing the gas pressure. Thus a set of zones containing foam fractions may be built up in the tube. In general, the stability of the foam decreases with each subsequent fraction, and eventually the residual liquid becomes so depleted of foam-stabilizing material that the treatment must be stopped. In any case, this stopping-point is reached when the gas pressure is such that eddies are produced in the foam column (3).

Sometimes, after some components of a mixture have been foamed out, there is a sudden increase in foam stability. Schütz (23) observed such an occurrence during the foam fractionation of a crude preparation of "sodium tauroglycocholate" at pH 6.5, initial concentration 0.4% to 0.5%, and a temperature of 24° to 25°C. A nitrogen pressure of 0.5 lb./sq. in. brought over a foam containing what appeared to be largely fatty acid (m.p. 58°C.). When the foam stability fell, pressure had to be increased to 4 lb./sq. in. to bring over another fraction; then 12 lb./sq. in for a third (m.p. 56°). All appeared to be fatty acid in nature. The foam stability then suddenly increased greatly, and the gas pressure had to be reduced to less than 0.5 lb./sq. in. The foam fraction that came over then looked as in Fig. XI-3,*C*, and contained crystals of what appeared to be bile acid, m.p. 188°, 190°, 191°C. In all these foams crystals appeared, indicating that concentration of the foamed substances occurred as well as enrichment in components.

Sometimes solid material is found in foam fractions owing, for example, to denaturation of protein or to flocculation of colloid. In other cases it may be crystalline, implying that surface orienting forces may aid in packing the molecules in a regular manner.

If the solute being separated is very surface-active, it can often be highly concentrated by foam fractionation. An example of this was given by Ostwald and Siehr (700). From 3 l. of an aluminum stearate sol, practically all the stearate could be concentrated into about 18 ml. of liquid (broken foam) in 11 min. of foaming with nitrogen.

Table XI-1 shows some foaming experiments, and indicates the wide variety of substances that have been examined.

An example of the use of *droplets* falling through a column of liquid as a means of producing a separation has been given by Strain (891). The plant pigments neoxanthin and fucoxanthin, which are water-insoluble and petroleum ether-soluble, contain hydroxyl groups that assist their accumulation at a petroleum ether–water interface. If water droplets from an atomizer are allowed to fall through solutions of these substances in petroleum ether, in a long narrow tube, the pigments are adsorbed to the interface of the droplets and are carried to the bottom of the tube, where they are set free as the droplets coalesce. The pigments are thereby concentrated in this region. Carotene, a hydrocarbon pigment,

TABLE XI-1
Some Substances That Have Been Foamed

Substance	Behavior	References
Acid phosphatase	Inactive proteins in foam	566
Albumin and protamine	Ovalbumin foams best at pH 4; salmin sulfate at pH 10	925
Bacteria	Koch bacillus in foam, and a white staphylococcus in residual liquid	236
Bacteria, tubercle	See Tubercle bacteria.	
Bacterial toxin	From *Clostridium septicum*, conc. and purified in foam at 28°C., pH 5.5	749
Bacteriophage	Some enrichment in foam	795
Catalase	Preparations in buffer, inactive protein in foam	564,565
Cholesterinase	Impurities foamed out at one pH, enzyme brought into foam at another, but destroyed	16,24
Diastase	Malt diastase concentrated in foam, pancreatic lipase left in residue by pH control	699
Fuchsine	See Night blue.	
Gonadotropic hormone	Concentrated from urine in foam	197
Hemoglobin	Does not foam from pure water or dilute salt; strong salt drives hemoglobin into foam	237,238
Lipase	See Diastase.	
Malt diastase	See Diastase.	
Methyl celluloses	Foam contains higher mol. wt., slightly more highly methylated fraction	832
Neucoccin	See Patent blue.	
Night blue	Sep'd. from basic fuchsine, night blue in foam, red fuchsine in residual liquid	252
Oleic acid	Purification of commercial	251
Ovalbumin	See Albumin.	
Pancreatic lipase	See Diastase.	
Patent blue	Sepd. from neucoccin. Patent blue in foam	698
Protamine	See Albumin.	
Resin acids	From fat acids	252
Salmin	See Albumin.	
Serum cholesterinase	See Cholesterinase.	
Sodium laurate	From stearate. At 45°C. foam enriched in lauric; at 90°C. foam enriched in stearic	252
Sodium oleate	From sodium laurate. Some conc'n. of oleate in foam	252,253
Sodium stearate	See Sodium laurate.	
Soybean protein	Maximum foam at isoelectric point	721
Staphylococci	See Bacteria; Tubercle bacteria.	
Tobacco mosaic virus	Larger particles made smaller in foam, increasing number of infectious elements	729,794
Tubercle bacteria	Separated from staphylococci	235
[illegible]	Concentrated into foam (CO_2)	388
[illegible]	Passed into foam from acetate buffer	565
[illegible]	Lapine-virus passed into foam	979
Virus, tobacco mosaic	See Tobacco mosaic virus.	

likewise insoluble in water and soluble in petroleum ether, but containing no hydroxyl groups, is not thus concentrated. If water droplets are made to fall through a column of solution of fucoxanthin and carotene in petroleum ether, the fucoxanthin can be taken off at the bottom of the column, being thus both concentrated and enriched. The process can be made continuous by injecting petroleum ether solution at the lower end of the tube, and withdrawing purified carotene solution at the top and a fucoxanthin fraction at the bottom.

V. VARIABLES

There seems to be general agreement that in order to produce successful separations certain variables must be controlled at optimum values. These seem to be concentration of the solutes, temperature, rate of bubbling and bubble size, pH (where relevant), and concentration of additives that may be used to decrease the solubility of the solutes.

1. Concentration of Solutes

Shedlovsky and co-workers (847), working with dilute solutions of detergents, recommend using that concentration that gives a minimum in the surface tension–concentration curve of the mixture to be foamed. But Schütz found little or no correlation between sudden changes in foam stability and surface tension changes. It would seem that the systems investigated by Shedlovsky and his co-workers represent a very special case wherein a highly surface active material is present in minute amount, in the presence of less surface-active micellar material, so that to make the best separations it is essential to choose a concentration at which the surface activity of the impurity is high. The best conditions are to be met at the concentration at which the surface tension–concentration curve shows a minimum (Fig. XI-6). This minimum occurs (10,368,577,636,847,848) in the region of "critical concentration for micelle formation." At this point and at higher concentrations micelles begin to appear in quantity, dissolving or sorbing the impurity that caused the very great lowering of the surface tension, thus *changing its chemical potential*. This causes the surface tension–concentration curve to rise up to the level of equilibrium between impurity-molecules and micelles. The existence of the minimum value in the curve, with slope zero, does not imply no adsorption but indicates the unsuitability of *concentration* measurements for judging what is occurring in the system. Such situations as these, though industrially and theoretically important where they occur, are probably not very widespread.

A simple procedure for determining optimum concentration, suitable for ordinary foam fractionation where there is little information about the contents or properties of a mixture to be treated, has been described by Schütz (832,834). This consists of constructing a foam stability–concentration plot, by standardized shaking of solutions of different concentrations, as described in Section II, and plotting the foam time T_f against the concentration of the solution. This necessary procedure may be

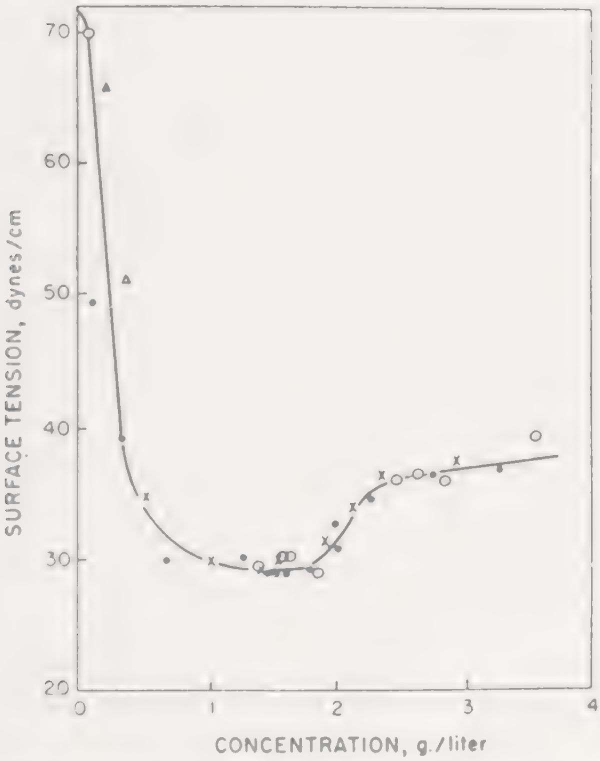


Fig. XI-6. Surface tension curve with a minimum. Surface tension of lauryl sulfonic acid, showing a curve with a minimum. The values were obtained by the following methods, at the times stated, and represent final lowering of the surface tension: (○) PLAWM trough (368) at 60 min.; (△) PLAWM trough at 10 hr.; (×) Wilhelmy method (368) at 45 min. at least; (●) ring method at 1 to 2 days; (▲) capillary rise method, time not stated. (After McBain and co-workers (578).)

tedious if the foams are very stable, but by raising the temperature of the systems the stability of the foam may be decreased to a convenient value. The general shape of the T_f–concentration curve is not thereby greatly altered, though the heights of maxima may be changed.

In general, foam stability vs. concentration curves will show one or more maxima. This applies to all kinds of solutes that foam. It is at a concentration value of a maximum in the foam stability that the foaming

experiment is carried out if the stabilizing substance is to be removed in the foam. Schütz suggests that in order to maintain this concentration it may be advisable to add concentrated liquid to the vessel from time to time through the stopper at the top (Fig. XI-3) to replace foamed-out material.

Where no maximum indicates the proper concentration to use, Schütz recommends foaming the solution of lowest practicable concentration since, as he points out, the surface-active substances will generally come close to saturating the interface in any case, and the entrained fluid, which is the chief source of impurities, will thereby be reduced in contaminating effect.

The ratio of surface-active components in the mixture affect foam stability, but the experimenter may have no control over this in the mixtures he has in hand. As an example, Schütz cites a mixture of bile salt and saponin in a ratio that does not give very stable bubbles. When this is foamed at a high pressure to whip the bubbles into the receiver before they collapse, several fractions can be taken off, and then suddenly there is a considerable increase in foam stability. This occurred when 95% of the bile acids had been removed (in the first fractions). At this point the remaining saponin could give a stable foam containing only traces of bile acid.

Solutions of large molecules also show an optimum concentration for foam fractionation. Thus London, Cohen, and Hudson (565) found a range of about 0.01% to 0.2% protein in buffer of isoelectric pH to be the best for foam purification of urease, catalase, and acid phosphatase preparations. The urease, which tended to concentrate in the foam, was best separated near the higher concentration end of the range (0.16% protein seemed best), whereas the catalase and phosphatase, which tended to be purified by having other proteins foamed away from them, were better purified near the lower end of the concentration range.

2. Temperature

The temperature of foaming may play an important role in the separation, either through affecting foam stability or through affecting the nature of the material appearing in the foam. An example of the latter effect is given by Dubrisay (252). When a mixture containing 1% sodium stearate and 1% sodium laurate in water was foamed it was found that at 45°C. the foam was enriched in lauric acid; at 90°C. in stearic acid. This was correlated with the fact that at these concentrations and at 45°C. sodium laurate lowers the surface tension of water by 35.5 dynes/cm. whereas sodium stearate lowers it by 25.2 dynes/cm. The laurate is thus more surface-active. At 90°C., however, sodium stearate lowers the

urface tension by 44.6 dynes/cm., while laurate lowers it by 21.19 dynes/ m., and the stearate is more surface-active.

When thermolabile substances are foamed it is desirable to use a low emperature, as suggested by Schütz (834).

3. Rate of Bubbling and Bubble Size

The effects of these factors are not thoroughly worked out. Schütz finds ubbles of small size to be advantageous in most cases. He finds that niformity of bubble size is important, particularly because foam staility is used as the guide to taking fractions and is dependent on bubble ize. Thus he finds sintered glass bubblers unsuitable. On the other hand, London and co-workers (565) recommend large bubbles in foaming roteinaceous materials. Possibly, with sensitive materials the denaturing ffect is greater with small more curved bubbles.

The rate of bubbling is extremely important. Slow bubbling, with lenty of time for the bubble to approach surface equilibrium with the olution, gave the best separations. In addition, the foam must be given ime to drain. Thus (834) when bubbles remained in the foam chamber on he average of 30 to 40 sec. before going over into the receiver (Fig. XI-3), concentration of bile acids up to six times that in the residual liquid vas obtained in the foam fraction, whereas if the bubbling speed vas increased so that the time in the foam chamber was only 5 sec., ardly any difference in concentration was found between residue and eceiver fractions. If, however, with rapid bubble flow or a multijet orifice he height of the foam chamber was increased to allow adequate drainge time, good separations and concentration were obtained. These obervations do, in fact, point the difference between Schütz's and Abribat's rocedures, described at the beginning of Section IV.

4. Hydrogen Ion Concentration

When salts, as of fatty or bile acids, are foamed, the foam contains free cid, and the residual liquid becomes alkaline if it is unbuffered. Apparntly, this is a manifestation of the tendency for undissociated acid to ccumulate more in the interface than the ion-pairs of the salt, since his latter would produce a fairly concentrated layer of ions.

When amphoteric substances are foamed it is found in general that the pH of the system has a marked effect on foam stability. In general roteins foam best at, or just on the acid side of, their isoelectric points (925). It was found also, with ovalbumin and salmin sulfate, that alkali arth ions or salts greatly promoted foaming in the alkaline range, but ninimized it in some cases on the acid side of the isoelectric point. Schütz

and co-workers (16,24) found that when serum cholesterinase was foamed at a pH where it did not enter the foam, impurities could be foamed away from it. When, however, the pH was such that the enzyme entered the foam, it was destroyed. Some proteins are denatured by foaming; others are stable to foaming.

5. Additives

Additives may be introduced to improve foam concentration or separation. The effects do not, however, seem to yield a clear pattern. A good working rule is that additives that decrease the solubility of the substance to be foamed will improve the separation. Thus Schütz (834) found that it was difficult to foam aqueous solutions of inulin; but that solutions containing 16.5% alcohol or 10% dioxane gave stable foams.

Abribat (3) has found the addition of *small* amounts of certain substances such as alcohol or acetone to solutions that foam such as proteinaceous solutions may greatly improve the stability of the foam. He interprets this as a dehydrating effect of the alcohol, itself surface-active, on the interfacial film, giving it rigidity. The presence of too much of these substances, he finds, diminishes the stability of the foam because the excess of surface-active alcohol or acetone displaces the rigidity-producing substances from the interface.

6. Drainage

Stability of foam is not in itself sufficient to guarantee good foam fractionation. Abribat has pointed out that a foam can be stable and yet the fluid between the bubbles can be so viscous that drainage is negligible. In this circumstance the concentration of substance in the foam is not significantly different (in terms of the volume of the liquid present) from that in the residual liquid. Examples are found in concentrated protein solutions—a familiar one in the foam of beaten egg-white. When situations of this kind are met, the liquid must be greatly diluted.

VI. GENERAL REMARKS

There seems to be no satisfactory quantitative theory of foam formation that is clearly applicable to foam fractionation, though many theoretical studies on foams have been made (74,637,716,832,833). There do seem, however, to be discernible patterns in the observed behavior of the systems that have been investigated.

Dubrisay, who has done pioneering work in this field, takes it as a working hypothesis that in a mixture of substances the one most active

in lowering surface tension will tend, other things being equal, to concentrate more relative to the other in foam produced from this solution (252). But the rule, which is exact when applied to a surface of a solution in equilibrium with solute, has many exceptions when applied to foam fractionation where equilibrium is not the rule, and factors such as rate of formation of bubbles, speed of drainage of the foam, foam stability, and associated factors such as dissociations, distributions between micellar and molecular states of solute, denaturation reactions, and differences in rates of diffusion, have an effect on the distribution of the materials present between bulk fluid and the interfacial film. In spite of all these qualifications, Dubrisay's working hypothesis remains a sound one.

To this may be added the corollary that any factor that drives a solute into the interface will aid foam or emulsion separation of that solute.

VII. MICROFLOTATION

Chromatography is restricted to the separation of molecular mixtures (Chapter I), but the line between these and mixtures of microscopic particles is not a sharp one, especially when the microscope is an electron microscope, and the molecules may be macromolecules, so that it requires not too great a stretch of our definition to justify the inclusion of a few remarks about the separation method that Dognon calls "microflotation" (235,236).

He has observed that suspensions of bacteria and yeast will foam and that certain organisms may be concentrated in the foam. For example Koch bacilli (B.C.G.) can be concentrated in a foam produced from their dilute suspension, but under similar conditions *B. coli*, a white staphylococcus, and a yeast do not concentrate appreciably in the foam, though there may be some entrainment.

Addition of foaming agents such as saponin and of salts (sodium sulfate, sodium chloride, calcium chloride) can drive all the organisms studied into the foam. By using these observations, separations of different types of bacteria can be brought about. For example, Koch bacilli can be foamed out of a sample of urine, and thus concentrated to an extent permitting identification. Then the residual fluid can be saturated with salt and foamed again, whereupon all other bacteria and all particulate matter will be concentrated in the foam. (See Table XI-1 for examples of separation or concentration of large molecules, viruses, etc.)

VIII. ESTIMATE

It must be acknowledged that the method of foam and emulsion fractionation is not yet well worked out from the point of view of predicting

what substances will foam and in what order. Too little is known of a factual nature from which to draw conclusions. For this reason the working rules given in previous sections must be taken provisionally. Yet there is enough evidence to suggest that the method can be a valuable one, and perhaps especially in the field of high molecular weight substances. In particular, because the *interface is homogeneous*, it may be that small differences between large molecules may efficiently be able to exert their effects on the bulk liquid–interface distribution. Further, the homogeneity of the "mobile" interface surely must assist in simplifying the problem of understanding what goes on in the process.

CHAPTER XII

On Recognizing and Evaluating Zones

I. INTRODUCTION

The formation of well-shaped discrete zones is an aim in most chromatography. Such zones in columns or on paper have well-marked boundaries and a regular shape. In effluent, whether gaseous or liquid, the aim is to obtain the zone component in a volume of fluid that is well defined by marked concentration change from preceding and following effluent. In all cases, the limits found for the zone are related to the limits of the tests or instruments used to discern the zone material. Thus the same zone may appear larger when a more acute test is used than it would appear to a less acute test, e.g., to an interferometric as compared with an Abbe refractometric examination.

In this chapter an attempt is made to show the various types of tests that have been used to find zones. It is beyond the scope of this work to discuss or even list all the tests that have been used for various kinds of zone materials. This is partly because there are so many of them. For example, in an appendix Hais and Macek (362) list something over 220 different tests and modifications of tests applicable to paper chromatography alone. The number is constantly increasing, and is fully as large in the area of adsorption chromatography. Further, these tests are to be found collected elsewhere (69,84,529), and only the broadest principles need be given here, as a check-list.

The same considerations apply to the quantitative evaluation of zones, which may be another objective of chromatography. Still another objective, equally important, is identifying the substances in the zones. As a strictly chemical problem, this does not belong here. Insofar as it concerns R and R_F comparisons with known substances it has already been discussed (Chapter V, Sections III and VIII; Chapter VI, Section VII; Chapter VII, Section X; Chapter VIII, Sections VII and VIII; and Chapter IX, Section X).

II. CLASSIFICATION

The methods for revealing or visualizing zones, or finding their limits, all may be classed as methods for detecting changes or differences in concentration. They may be nonspecific or specific. They may apply to

stationary phase and to eluent, or to only one of these, and they may be physical, chemical, or biological in type. All these methods may be made quantitative—at least to some extent; and some of them can be both quantitative and highly specific.

The methods classed as applying to the stationary phase apply to columns while inside a chromatography tube or after extrusion from the tube. They apply also to paper strips and sheets. Those applicable to effluent refer to effluent from columns, or to material eluted from strips or sheets, or pieces of them.

It is necessary to distinguish between sensitivity, which may be very high for a colorimetric or biological method, and quantitative results which may not be outstanding. It may, indeed, be that in many cases lowered accuracy is a small price to pay, if it has to be paid, for greatly increased sensitivity and specificity.

III. QUALITATIVE METHODS

1. Physical Methods

A. ELECTROMETRIC METHODS

Electrometric methods, applicable to zone recognition are dielectric measurements, measurements of conductivity, dipole moment (257), acid-base (820), oxidation-reduction, precipitation and polarography parameters, and high-frequency densitometry (372). Some of these can be applied to the stationary phase while it is inside or outside the tube, and all of them are applicable, under the appropriate conditions, to the effluent. Instruments have been designed to measure change in dielectric properties in a column as a zone passes along it and to measure changes in conductivity (243,935). The other measurements are more easily made in the effluent as it passes out of the tube. Examples of some of these measurements have been given elsewhere. For example, for acid-base measurements, see Chapter VI, Sections VI and VII (also Fig. VI-5). Techniques such as these are directly applicable to oxidation-reduction measurements. Conductivity measurement is used routinely to control demineralization of water with ion exchanger beds (511a,676). Precipitation tests and polarography (244,529,614) are frequently used in chromatography of inorganic substances and are applicable also to organic. A pH meter of the flow type may be attached at the foot of a column, or the effluent may be collected in fractions and analyzed by any of the above methods.

B. GRAVIMETRIC METHODS

These are the most widely applicable methods. They are not useful in connection with adsorbent inside a column but are applicable to paper in the sense that a spot may be cut out and weighed to derive its area. Their greatest usefulness is in connection with the empirical treatment of an extruded column and in the examination of effluent.

If no other method for finding the zones of a material on a column is practicable—as might be the case with a mixture of quite unknown composition or with a mixture of inert substances—an approach to their chromatography is as follows. Frontal analysis of a small amount of the mixture is carried out with several adsorbents and developers, as outlined in Chapters XIV and XV. This gives information about the capacity of the adsorbents for the least strongly adsorbed component of the mixture. A column is made up containing 10 to 20 times as much adsorbent as will just retard a certain amount of the mixture from a known volume of solution. This amount of mixture is applied to the column and developed until material other than developer appears in the effluent. At this point it may be inferred that the mixture is spread out along the column. The column is then extruded from the chromatography tube and cut into sections. The number of sections taken is governed by one rule: there should not be so many taken that the amount of substance in each of them is too small for accurate measurement. Some sections which happen to come between zones may contain little or no adsorptive, but those that comprise zones should have measurable amounts of adsorptive on them. The sections are then packed into smaller tubes and the adsorptive is eluted with a solvent or mixture separable from it by some means; the adsorptive is recovered and weighed. When the amounts of adsorptive per unit length of section are plotted for the column, peaks of concentration will indicate zones. Another column, run in the same manner can then be cut between the peaks. (See Chapter XV for details.)

Gravimetric analysis of the effluent follows the same principles. Fractions are collected and the adsorptive is recovered by evaporation of the solvent or by some other means. A plot of grams per unit volume against number of fraction (if the fractions are of equal volume) or against volume, yields peaks at the positions of zones.

C. MARKING SUBSTANCES

Marking substances may be of two general types: those that adhere to the stationary phase and indicate the location of a zone as it goes along

the column, and those that precede, follow, or otherwise bear a definite relation to the zone in its development along the column and into the effluent.

Indicators of the first type have been used in column and paper strip chromatography. For example, Graff and Skau (339) separated stearic from oleic acids on a column of magnesium oxide colored with phenol red. Petroleum ether was used as the developer. The indicator changed from pink to yellow in the regions of the zones. Sylvester, Ainsworth, and Hughes (904) tinted alumina with bromothymol blue, and used such columns to separate traces of fatty acid from unsaponifiable matter. The developer was chloroform or ether. Bromocresol green is commonly used in the stationary phase of partition columns as an acid indicator (see Table VI-2).

Fluorescent adsorbents were prepared by Brockmann and Volpers (119,125) and by Sease (842,843) (among others). The former workers adsorbed fluorescent substances onto their adsorbent: salicylic acid, etc., onto alumina, berberine onto silica gel; the latter mixed fluorescent pigments such as zinc sulfide into the adsorbent. Zones of certain colorless, nonfluorescing adsorptives could be observed where they quenched the fluorescence of the adsorbent. A similar technique has been used in paper chromatography (621). J. W. Green (342) converted sugar acids to their anilides, and chromatographed them with 9:1:2 (v/v) acetone–water–benzene containing 4 mg. Rhodamine B dye per 100 ml. After development the paper was dried and examined by ultraviolet light. The anilides, quenching the fluorescence of the dye, show up as dark spots on a light yellow background.

Indicators of the second type may be divided into two classes: those present in the mixture and those added for a particular purpose. In either case, the function of the indicator is to form a visible zone that moves along the column in a fixed relation to invisible zones of desired materials. An example of the former class was a colored zone found to move in a fixed relation to a penicillin zone. This fortuitous indicator was made use of in following development. An example of the latter was provided by Brockmann. He found (112,114) that the dye Sudan III appeared at the same place as vitamin D when it was mixed with a tuna-fish liver oil concentrate and was chromatographed on alumina with a benzene–petroleum ether (1:4) developer. The marked zone could be separated, the mixed adsorptive eluted, and the dye removed by extraction with alkaline methanol.

D. MEASUREMENTS OF P, V, OR T

Measurements of P, V, or T are particularly applicable to gas chroma-

ography and are dealt with in Chapter V. It may be feasible to follow the passage of a zone by temperature measurements in a column because often the process of adsorption releases considerable heat.

E. OPTICAL MEASUREMENTS

Optical measurements comprise color, fluorescence, index of refraction, infrared, optical rotation, turbidity, ultraviolet measurements, and possibly others. Not all are readily applicable to the stationary phase. Colored and fluorescent zones need no special mention. Sometimes a zone on a colorless but translucent adsorbent may be observed as a shadow, where refraction is changed by the presence of an adsorptive. For example, Trappe (943) observed that silica gel columns became translucent when wetted with certain solvents, and under these conditions it was possible to observe zones formed by colorless substances. Similar effects are often observed with ion exchangers (674,675).

The use of optical measurements of effluent concentration is well worked out for qualitative as well as quantitative analysis, and instruments are available for such applications (see Appendix). The first elegant instrument of this kind was Tiselius and Claesson's interferometer (927) by means of which the refractive index of the effluent in a reference cell was compared with that of empty developer or effluent. The appearance of a zone caused a change in refraction that could be registered manually or automatically. (See Fig. VIII-8.) Holman (413) introduced the technique of "crossing over," by means of which the range of the instrument could be extended. When the difference in index of refraction between the pure developer and the effluent approached the limit of the instrument, the pure developer could be replaced with some of the effluent which was temporarily bypassed into the reference cell. This gave a new base line for comparison of subsequent effluent.

The modern automatic *ultraviolet fluorescence and other scanners* can be attached by appropriate means to the foot of a column to examine effluent (68,124,125,582,583,786). One such device has been named a "scanalyzer" (467a). Turbidity measurements may be made on effluent fractions as part of precipitation or bacteriological analyses.

F. RADIOMETRIC AND ISOTOPIC ANALYSIS

Radiometric and isotopic analysis have been developed to a high degree of specificity and accuracy, particularly in the inorganic and biological fields. Columns and paper strips may be scanned by various devices that detect α, β, or γ radiation, or strips or sheets may be dried and placed in contact, in terms of radiation, with photographic film or

paper, whereupon a *radioautograph* may be produced (468,670,788). It is important in such work to exclude the possibility of *chemical* development of the film or paper by reagents in the paper, or by debris from pencil marks, etc. on the paper (354,748). Lastly, a developed chromatogram may be made radioactive by neutron activation and then differences between the activated spots and the (weakly) active paper can be looked for by scanning or autoradiography (1016).

2. Chemical Methods

More limited in applicability than many of the physical methods, the chemical methods may for this reason be very specific. Color tests are applicable to the stationary phase and to the effluent. It is unfeasible to list all such tests. Many are collected in other books on chromatography and on chemical analysis (282a). Only a few examples will be mentioned.

A. BRUSH TECHNIQUE

The brush technique of Zechmeister (775,1036) involves streaking the extruded column with a color reagent which by its specific reaction shows the location of zones. These may be sculptured out from the column, the reacted, streaked adsorbent scraped off, and the remaining adsorptive recovered by elution. A slotted column (206) which could be opened in a narrow strip along its length was designed so that the course of development could be followed. The column could be opened, and a few spots of color reagent, applied with the tip of a brush, would locate the active material, and if development had not progressed far enough the column could be closed and development continued, with further testing if necessary.

B. COLOR REACTIONS

Paper strips and sheets are probably most often examined through color reactions. For example, in their first papers Martin and his coworkers revealed the spots of amino acids on papers by drying, spraying with ninhydrin, and heating. Since then many hundreds of specific and nonspecific tests have been applied to papers and sheets.

Color reactions have been applied to the effluent for qualitative and quantitative purposes. One of the best worked out of the latter is Stein and Moore's ninhydrin method for amino acids and related substances. By careful attention to detail they have evolved a highly accurate, reproducible, and reliable method (see below, and Fig. IX-4).

C. GASOMETRIC REACTIONS

Gasometric reactions may be applied to effluent for purposes of qualitative and quantitative analysis. Examples of the former might be the recognition of acid through release of carbon dioxide from sodium bicarbonate, of the latter, the analysis of amino and other compounds by one of the Van Slyke methods.

D. INDICATORS

Titrations with indicators or the use of indicators as reagents, is, where applicable, a convenient way of finding zones. A piece of damp indicator paper may be laid along an extruded column, for example, or small pieces may be used to test effluent. For analytical purposes a device such as that designed by Martin (Fig. VI-5) is very convenient. It is applicable to volumetric analysis of effluent.

Colored zones need no particular mention.

3. Biological Methods

A. ODOR AND TASTE

Odor and taste may be used to follow break-through experiments, as in water purification (53,374). The invention of gas partition chromatography will lead to a rapid and broad development in the field of odor and taste research, since the method is tailor-made for the study of natural perfumes, sex attractants, and so on.

B. BIOLOGICAL ACTIVITY

Perhaps the most specific way of testing for zone substances where it is applicable is through biological activity.

Adsorptive actually on the adsorbent may in some cases be fed to test animals with subsequent response (420). However, normally the active material must be removed from the stationary phase or must become accessible from the stationary phase. An elegant method for testing growth antagonists and growth stimulants was introduced by Goodall and Levy (231,332). The developed chromatogram, as a strip (developed as a strip or cut from a sheet after development), is laid on a plate of nutrient substrate, seeded with an appropriate organism (or mixture of organisms). After a period to allow the zone material to diffuse into the nutrient medium the plate with the strip still in place, or with the strip removed, is incubated. Stimulated or depressed growth at certain regions along the strip gives evidence of zones of stimulant or of antagonist,

respectively. The area of a zone may be used to get a semiquantitative estimate of the amount of active material present. Alternatively, the strip or sheet may be cut into pieces and these incubated in broth or on plates. Fractions of eluent may also be treated in this way, with subsequent quantitation, if desired (84).

IV. QUANTITATIVE METHODS

1. General

A great deal of progress has been made in developing quantitative chromatography. The most spectacular successes have perhaps been achieved with radioactive tracers and radiometric instruments, especially in the area of ion exchanger separations. However, other methods are running these a close race: for example, Moore and Stein's methods for amino acids (Chapters VII and IX) and Martin and James' and others' methods in gas partition chromatography (Chapter V).

Most of the highly quantitative methods are carried out in effluent or on materials eluted from zones. Quantitative analysis of material in spots or zones on paper has been highly developed. Block, Durrum, and Zweig (84) have given detailed and critical discussion of these methods.

The factors that seem most to hinder quantitative analysis (apart from instrumental ones) are of two kinds: nonideal behavior of adsorptives, because of which the zone material does not move in a compact zone but trails out behind or in front of the main zone; and background impurities in the paper or other supports that affect the analyses. Attacks on problems of the first kind have been actively made by Tiselius and his coworkers (see Chapter VIII), leading to the invention of displacement analysis and carrier displacement, and gradient analysis combined with blocking of excessively active sites on the adsorbent ("saturation"). By these expedients tailing has in many cases been brought under control and excessively curved isotherms have been somewhat straightened. Problems of the second kind are also subtle and comprise, for example, not only the presence of amino acids in paper (242), which in delicate work may be very trying, but the unknown causes of loss of material (923) (when there is so little to lose) and the known catalytic effects that may occur on adsorbents or in developers (Table VIII-9).

Observations made by Shapiro (844a) are important in this connection. He found from two to six water-extractable, ninhydrin-positive substances in Whatman filter papers numbers 1, 3, 40, and 54. Methods tried for their removal from No. 3 paper included chromatographic washing with water for up to 176 hr.; washing with acetic acid followed by water, followed by ammonia; treatment with either nitrous acid or ninhydrin

followed by washing with water. None of these methods worked completely. The substances could be reduced to a low level temporarily, i.e., the second elution yielded less than the first. But after the paper had been allowed to stand, the next elution yielded the material in larger quantity than the last elution of the previous series of elutions. Also, substances reacting with *p*-anisidine have been detected in Whatman No. 3 paper. It is possible that these traces of undesirable substances occur within the fibers, and slowly diffuse out as the surface is washed off.

Shapiro (844a) reports also that elution with some developers may give rise to misleading results if the developers are not previously purified rather carefully. He found that A. R. formic acid contained a ninhydrin-positive substance which remained in the residue during distillation. Tertiary butyl alcohol contained ninhydrin-positive material which was volatile. Mixtures of these liquids used to wash papers seemed to increase the background ninhydrin color of the paper. No difficulties of these kinds were encountered with methyl ethyl ketone–propionic acid developer.

Observations of these kinds make it necessary to scrutinize any experimental work reporting traces of substances found through paper chromatography. The paper must be rigidly excluded as a source of these traces.

It should be pointed out that for quantitative work the amount of material put on a column or paper should be accurately known. With the micropipettes and microburettes available (see Appendix) this does not present a problem. Further, the collection of fractions from the column must be accurately done, and again this does not present a serious problem since many such instruments are available (see Appendix under Fraction Collectors). With some reagents the quality of solvent is important. For example, Miss Dowmont (242) found that water demineralized with ion exchange resins was not suitable for quantitative ninhydrin tests since it gave blank values two to three times higher than distilled water—presumably because of traces of amines liberated from the resins.

In this section the various quantitative approaches are classified. As analytical problems they cannot be dealt with here in the necessary detail. The classification follows exactly that of the qualitative methods and presumably all of those could be made more quantitative.

2. Analysis of Zones in a Column

Analyses of zones in a column by measuring the length of the zone do not ordinarily yield quantitative results. That it is possible to make the method quantitative has, however, been shown in the case of the analysis of petroleum fractions for aromatics, olefins, and saturates, according to

the method of Criddle and LeTourneau (204). A long narrow column of standardized silica gel is used. The sample, containing traces of fluorescent dyes, is applied, and displaced with an alcohol displacing agent. At complete development the separated fractions, the zone of each of which is marked by a dyestuff component, are ranked: saturates in front, followed by olefins, followed by aromatics, followed by displacer. The lengths of the zones are proportional to the concentrations of the components in the original sample, with reproducibility of about 1% to 2%. The method has been extended to other organic mixtures by Knight and Groennings (483).

3. Size of Colored Spots

The size of a colored spot on a paper chromatogram has been found to be related to the amount of material in it (84). Studies of this kind have been going on for some time (170). Applied to chromatograms, visual comparisons, measurements of length of zones (653), and measurements of area of spots, have been made. In the last of these, the area, instead of being measured with a planimeter, may be determined from the weight of the paper after cutting out the spot. Other techniques use the total color density of the spot, the maximum color density, the area times the density, or the reflectance of the spot to correlate with concentration. In addition, the spot may be photographed or radioautographed and a technique such as one of those listed may be applied, or the spot if radioactive may be examined radiometrically, and thus analyzed for radioactive components. Combinations of the methods are frequently most useful. For example, a tissue extract incubated with radioactive triiodothyronine was one-dimensionally chromatographed with known nonradioactive reference substances (monoiodotyrosine, thyroxine, etc.), and then visualized with a color reagent, diazotized sulfanilic acid. A densitometer plot of the color spots, superimposed on a densitometer plot of an autoradiograph of the same chromatogram, showed which of the colored spots contained radioactive iodine, and thus aided identification of the products in the incubated extracts (354).

4. Analysis of Eluents

Quantitative analysis of eluent fractions has already been mentioned. Stein and Moore (648) analyzed amino acid fractions with a ninhydrin method sensitive to 1 p.p.m. amino acid in aqueous or alcoholic solution (about 150 times the sensitivity of refractometric measurement to $2 \times 10^{-5} N_D$). In analyses of mixtures containing 19 components a single determination of a component present to the extent of 3% or more in the

mixture was seldom in error by as much as 5%, and the average of several determinations gave accuracies of ±3%. Similar accuracies were obtained using starch and ion-exchanger columns (654). Claesson reports high accuracies for the continuous interferometric analysis of effluent by his method (177). For example, a mixture of normal C_8, C_{10}, C_{12}, and C_{14} acids, 0.2 g. total, at a concentration of 0.469% in absolute ethanol was analyzed by frontal analysis, and each component was determined with 5% accuracy or better. The self-recording interferometer used is capable of determining refractive index to better than 10^{-5}. The instrument, when used by counting fringes, has a range of Δn (change in refractive index) of 2×10^{-3} to 3.2×10^{-4}, depending on compensator settings, and an accuracy of *ca.* 1×10^{-6} to 5×10^{-7} at these settings (935).

In general, it may be concluded that the quantitative side of chromatography is well developed. At least, the principles are well established, and the instrumentation is highly advanced. The part of chromatography most in need of sound principles and reliable generalizations is that which precedes quantitation, namely, the choice of a system that will bring about an optimum separation of the mixture in hand.

On the Relation of R or R_F to Molecular Structure

I. INTRODUCTION

The problem of evaluating and predicting the interactions that molecules may exhibit, for example, as they are reflected in the way molecules of a solute may distribute between two phases, has always intrigued chemists. Thermodynamic approaches have been made to it, as well as more qualitative approaches that begin with structural considerations. The former are of the greatest value in making available a theoretical scaffold (at least) upon which the practicing chromatographer, working with inadequate data as he usually is, can hang his observations and chemical intuitions. The latter are often more easily bent to use.

II. LANGMUIR'S ANALYSIS

One of the earliest of these approaches was made by Langmuir (521). Study of the *energy of transfer* of a molecule from one phase to another, as in adsorption, evaporation, and so on, led him to postulate that in so far as it involved breaking contacts with other molecules it could be subdivided into parts related to the per cents of the "surface energy" of the molecule represented by its chemical groups, such as $—CH_2—$, $—OH$, $—COOH$. The concept, termed the "principle of independent surface action" has been tested and found to contain interesting possibilities (866,956,957). Cohn and Edsall have used somewhat the same ideas in treating solubilities of amino acids (189).

III. DISTRIBUTION BEHAVIOR

At least three groups of investigators have approached this problem in recent years: LeRosen and his co-workers, Martin and his co-workers, and Pierotti and his co-workers.

Practically speaking, it may be allowed that the R or R_F value of a substance, which is what the chromatographer wishes to control, is some function of A_M, A_S and α, where A_M and A_S are cross-sectional areas of the mobile and stationary phases and α the distribution coefficient (see Chapter IV). As a general rule, it would seem, that R and R_F can be influenced more easily and markedly through α than through A_M or A_S. This is why the accent is placed on the distribution coefficient.

The distribution behavior of a solute between two phases has been correlated with many properties of the system (402). A common correlation has been based on solubility: on the consideration that if two phases (S) and (M) are each saturated with a component 1 in equilibrium with solid component 1, then

$$C_1^{(S)}/C_1^{(M)} = S_1^{(S)}/S_1^{(M)} = K,$$

where C represents concentration, S solubility, and K is a constant. However, most systems of interest to the chromatographer are far from saturation—at least in the mobile phase—and also, the solubility of a component in a given solvent is often markedly affected by the presence of other components. In spite of the numerous exceptions to any quantitative application of this correlation it does, in fact, remain a very useful qualitative guide. Lacking more specific information it may, for example, be assumed that in a given mobile phase the more soluble components of a mixture will show higher **R** or $\mathbf{R_F}$ values than the less soluble, provided they do not react chemically with the stationary phase. Considerations of this nature have already been discussed in earlier chapters, and will be reverted to in Chapter XIV.

IV. LEROSEN'S APPROACH

LeRosen and his co-workers (548) took a partly empirical approach to the problem of adsorptive distribution. They were interested in the correlation of molecular structure with the **R** values of adsorption chromatography. The most important properties of adsorbent, developer, and adsorptive for this purpose seemed to be electron donor, acceptor, and hydrogen-bonding strength, and dispersion interactions (discussed in Chapter III). These could be evaluated for a number of solvents, adsorbents, and adsorptives by measuring the **R** values for various combinations of solvents, adsorbents, and adsorptives. Arbitrary reference standards were set up for the different interactions using separate scales of values for adsorptives, adsorbents, and developers (see Tables XIII-1, 2, and 3). Values for the different factors were calculated to agree with the observed **R** values.

For example, in evaluating the interactions of adsorptives, the unshared pair of electrons of a tertiary alkyl amino nitrogen, such as triethyl amine $(C_2H_5)_3N$:, was defined as representing one electron donor unit (D = 1.00). The acceptor unit was defined as the affinity of a trialkyl boron, such as tributyl boron, for a pair of electrons (A = 1.00), and the unit of hydrogen donation in hydrogen bond formation was assigned to the hydroxyl hydrogen of a primary aliphatic alcohol, R—CH_2—OH, (H = 1.00).

Among developers, petroleum ether was assigned A, D, and H values of 1 each. Then, by using a given adsorbent and adsorptive with petroleum ether as a standard, and again with another developer, such as benzene, it was possible to determine relative values of interaction tendencies of the other solvent (benzene) relative to petroleum ether.

A number of such interaction tendencies are given in Table XIII-3. The authors regarded these as subject to revision and stressed that the *scales* of evaluation for developers are different from those for adsorbents.

TABLE XIII-1

Nomenclature[a] with Rationalization of the Symbols

R	= the ratio of rate of movement of the adsorbed compound in the column to the movement of the developing solvent in the column; $\mathbf{R}_L$ applies to the front edge of the zone, $\mathbf{R}_T$ applies to the rear edge.
	= the proportionality factor in the adsorption isotherm such that the amount of substance adsorbed on the adsorbent in equilibrium with one unit volume of solvent is obtained by multiplying the concentration in solution by f. The value of f may vary with concentration.
[illegible]	= an equilibrium constant for the adsorption reaction.
[illegible]	= surface area of the adsorbent in terms of moles per unit of adsorbent as defined for f.
T_M	= statistical average time an adsorptive particle spends in solution between adsorptions.[b]
T_S	= statistical average time an adsorptive particle spends on the adsorbent during each adsorption.[b]
M	= applies to the sum of the molecular weights of all side chains in an adsorptive molecule.
D	= donor strength of substance in respect to electron pair. D_S refers to the adsorbent; D_M, to the developer; D_a, to the adsorbed compound. These subscripts are also applied similarly to terms listed below.[b]
A	= acceptor strength of a substance for an electron pair.
D^H	= donor strength in terms of an electron pair donated to a hydrogen atom in hydrogen bond formation.
H	= acceptor affinity of a hydrogen-bonding hydrogen for an electron pair.

[a] From LeRosen, Monaghan, Rivet, and Smith (548).
[b] Note: T_M and T_S are T_S and T_M in LeRosen's paper; D_S is D_a, D_M is D_S, and D_a is D_S.

TABLE XIII-2

Rules for Calculation of **R** Values (548)

1	In aromatic amines the donor strength of the nitrogen was divided by the ratio of the total number of most important resonance forms to those showing the electron pair on the nitrogen atom.
2	An internally bound hydrogen (to hydrogen bond) was disregarded; this included the acid hydrogen in case of dimers.
3	The H_S interaction of the adsorbent was not isolated and is included in the A_S value; therefore, this term is dropped from adsorption affinity calculations.

TABLE XIII-3
Interaction Tendencies of Substances[a]

	A	D	D^{H}	H
Developers				
Petroleum ether (purified Skellysolve B)	1[b]	1[b]	—	1[b]
Benzene (thiophene-free)	25	5.8	—	4.3
Adsorbents[c]				
Special Filtrol	14,000	1,333	1,300	—
Merck reagent silicic acid	4,800	2,570	120	—
Florisil	2,000	1,160	260	—
Merck heavy powder calcium carbonate	33	224	26	—
Calcium acid phosphate dihydrate	77	—	42	—
Magnesium oxide	47	3,350	190	—
Calcium hydroxide	40	11,500	23	—
Adsorptive				
Amino N (R_3N:)	—	1.00[b]	—	—
Alkyl B (R_3B)	1.00[b]	—	—	—
Alcohol H	—	—	—	1.00[b]
Alcohol O	—	0.17	—	—
Acid or ketone O	—	0.20	—	—
Nitro group	—	0.04	—	—
Aromatic ring	—	0.002	—	—

[a] From LeRosen, Monaghan, Rivet, and Smith (548).
[b] These values are arbitrarily given.
[c] The values may need to be redetermined for different batches of adsorbents.

and these different from those for adsorptives. Thus the hydrogen donating unit, $H = 1.00$, is assigned to a primary alcohol when it is an adsorptive. This same number but not the same *value* is assigned to petroleum ether as a developer. The scales must be different when the same number is given to two such chemically different substances as alcohol and petroleum ether for a parameter like hydrogen-bonding tendency. The scales are, however, consistent among adsorbents, among developers, and among adsorptives, and are related through the process of evaluation.

The equation used by LeRosen and his co-workers was set up on the basis of the following considerations. In the chromatography of a solute the substance is distributed between a mobile phase (the developer) and a stationary phase (the interfacial phase at the surface of the absorbent) according to some function which may not be a constant. As an approximation it may be taken that this function does not change with concentration, and that in the process under consideration a sufficiently close approach is made to equilibrium. A constant temperature is maintained. Then using the nomenclature of Table XIII-1,

$$f = ks = T_S/T_M = (1 - \mathbf{R})/\mathbf{R}$$

The statistical average time that a molecule of adsorptive spends in the stationary phase (T_S) relative to the time spent in the mobile phase (T_M) will depend as a first approximation upon the magnitude of the interactions that occur between it and the molecules of these phases. The **R** value is related to these times thus: $\mathbf{R} = T_M/(T_S + T_M)$ (542); that is, the rate of movement of the zone will be related to the fraction of the time that the molecule spends in the mobile phase (see Chapter IV). From these considerations the hypothesis was set up that **R** is related to the more important interactions, thus:

$$f = \frac{(1 - \mathrm{R})}{\mathrm{R}} = (M_{sc})^{-1}\left[\left(\frac{A_sD_a}{D_M}\right) + \left(\frac{D_sA_a}{A_M}\right) + \left(\frac{D_s^HH_a}{H_M}\right) + \left(\frac{H_sD_a^H}{D_M^H}\right)\right] \qquad (1)$$

Competition between adsorptive and developer for the surface of the adsorbent is clearly recognized in this formulation.

An example of the use of this equation in calculating the **R** value of ethylaniline on Florisil developed with petroleum ether is given by Le-Rosen:

$D_{NH_2} = 1$ (Fraction showing electron pair on nitrogen in resonance structures $= {}^1/_4$; for ethylaniline use $D_a = 0.25$)
H_s for one hydrogen $= 1$
$M_{sc} = C_6H_5 + C_2H_5 = 77 + 29 = 106$
D_M for petroleum ether is by definition 1
H_M for petroleum ether is by definition 1
A_s for Florisil was determined as 2000
D^H for Florisil was determined as 260

$$\frac{(1 - \mathbf{R})}{\mathbf{R}} = \left(\frac{1}{M_{sc}}\right)\left(\frac{A_sD_a}{D_M} \times \frac{D_s^HH_a}{H_M}\right) =$$

$$\left(\frac{1}{106}\right)\left(\frac{2000 \times 0.25}{1} \times \frac{260 \times 1}{1}\right) = 7.15$$

$\mathbf{R} = 0.12$

The experimentally determined **R** value is 0.13.

Using this relationship quite good agreements between calculated and experimental **R** values were obtained for two developers, seven adsorbents, and twelve or so adsorptives. Where the agreement was not good the

reasons were usually apparent. Thus the R values calculated for phenol on calcium hydroxide from petroleum ether, and benzene, are, respectively, 0.72 and 0.92. The observed values are in each case less than 0.01. Salt formation is thought to be the cause of the discrepancy. It would greatly retard a zone of phenol if a calcium salt were formed with the surface of the adsorbent.

Steric effects were not taken into account in setting up the equation. Later work by Smith and LeRosen (862) with *n*-alkyl methyl ketones from C_3 to C_{18}, some branched-chain alkyl methyl ketones, and cyclic and aromatic ketones, showed that many effects were not taken account of in the equation (1). It seemed that the exponent of the term $(M_m)^{-1}$ could not be simply -1, but would have to be made a function of the absorbent, the branching, and possibly of the particular homologous series. An investigation of disubstituted benzenes confirmed the complexity of the problem of relating structure to adsorbability (547). While among the nitrophenols, nitroanilines, chlorophenols, hydroxy phenols, amino phenols, cresols, anisidines, hydroxy biphenyls, hydroxy benzoic acids, and amino benzoic acids, the *ortho* compounds were in every instance less strongly adsorbed than the *para* onto silicic acid from benzene, the calculated R values did not agree with the experimental in a large number of instances. There were indications that steric, inductive, and perhaps other factors were in operation.

This approach has interesting possibilities, and LeRosen would, no doubt, have pursued it in less empirical directions had he lived to continue it (546). Very likely it could be generalized to any kind of distribution.

V. MARTIN'S APPROACH

The following description of this attack on the relation of distribution behavior to structure is taken from A. J. P. Martin (613). "If we restrict our discussion to ideal solutions, i.e., those obeying Raoult's law,

$$\mu_A^S = \mu_A^{S_0} + RT \ln N_A^S,$$

where μ_A^S is the chemical potential of the substance A, $\mu_A^{S_0}$ is the chemical potential in some defined standard state, and N_A^S is the mole fraction of A in the phase S.

"If two phases S and M are in equilibrium the chemical potential of all components is, of course, the same in each. Thus

$$\mu_A^M - \mu_A^S = 0 = \mu_A^{M_0} - \mu_A^{S_0} + RT \ln N_A^M - RT \ln N_A^S,$$

or if

$$\mu_A^{S_0} - \mu_A^{M_0} = \Delta\mu A,$$

$$\Delta\mu_A = RT \ln (N_A^M / N_A^S).$$

N_A^M/N_A^S is the partition coefficient (expressed in terms of mole fractions) = α.

$$\ln \alpha = \frac{\Delta\mu_A}{RT}$$

and $\Delta\mu_A$ is equal to the free energy required to transport one mole of A from phase S to phase M.

"Now to a first approximation $\Delta\mu_A$ may be regarded as made up of

$$d\Delta\mu_{CH_2} + e\Delta\mu_{COO^-} + f\Delta\mu_{NH_3^+} + g\Delta\mu_{OH} + \ldots, \text{ etc.,}$$

the sum of the potential differences of the various groups of which the molecule A is composed. That is to say, to a first approximation the free energy required to transport a given group, e.g., CH_2, from one solvent to another is independent of the rest of the molecule. Thus all isomers containing the same groups (note that the degree of ionization, etc., must not be changed) would be expected to have the same partition coefficient.

"Now, if we consider the partition coefficients α_A and α_B of two substances A and B which differ in that B contains, in addition to those contained in A, a group X, we have,

$$\ln \alpha_A = \frac{\Delta\mu_A}{RT}, \ln \alpha_B = \frac{\Delta\mu_A}{RT} + \frac{\Delta\mu_x}{RT}, \ln\left(\frac{\alpha_B}{\alpha_A}\right) = \frac{\Delta\mu_x}{RT}$$

"Thus the addition of a group X changes the partition coefficient by a given factor depending on the nature of the group, and on the pair of phases employed, *but not on the rest of the molecule.*

"This is a prediction contrary to the usual expectation. It is usually felt that the formation of a derivative of greatly increased molecular weight will 'swamp' any differences that exist and will render separation more difficult. This, however, is not to be expected if such a derivative be chosen that the same pair of phases can be employed while still maintaining convenient values for the partition coefficients.

"Let us apply this rule to amino-acids and peptides. On the formation of a dipeptide molecule from two amino-acid molecules, or a tripeptide from an amino-acid and a dipeptide, one —CONH— group is created and one COO^- and one NH_3^+ are destroyed. Let the amino-acids be $A_{COO^-}^{NH_3^+}$ and $B_{COO^-}^{NH_3^+}$ and the peptide $NH_3^+ \cdot A \cdot CO \cdot NH \cdot B \cdot COO^-$, and let the partition coefficients be α_A, α_B and α_{AB}, respectively,

$$RT \ln \alpha_A = \Delta\mu_{NH_3^+} + \Delta\mu_A + \Delta\mu_{COO^-},$$

$$RT \ln \alpha_B = \Delta\mu_{NH_3^+} + \Delta\mu_B + \Delta\mu_{COO^-},$$

$$RT \ln \alpha_{AB} = \Delta\mu_{NH_3^+} + \Delta\mu_A + \Delta\mu_{CONH} + \Delta\mu_B + \Delta\mu_{COO^-},$$

$$RT \ln \left(\frac{\alpha_A \alpha_B}{\alpha_{AB}}\right) = \Delta\mu_{NH_3^+} + \Delta\mu_{COO^-} - \Delta\mu_{CONH}.$$

i.e., the product of the partition coefficients of the constituent amino-acids divided by the partition coefficient of the dipeptide is a constant for any given phase pair.

"The predictions of this rule hold nearly as well as that for isomers. Thus a peptide AB should have the same value as peptide BA, and, in fact, they seldom differ by as much as 30%, while the range of partition coefficients covered by the rule may be very large indeed (factors of thousands)."

Tests of this theory have given it strong support. Bate-Smith and Westall (43) examined the $\mathbf{R_F}$ values of a number of hydroxy, methoxy, and carboxy derivatives of benzene. The $\mathbf{R_F}$ values were determined under careful temperature control, with 3-day equilibrium of the mixed solvents and 1-day equilibration of the paper strips. The developer was allowed to run 30 to 35 cm. and a control substance was run on each strip. If the $\mathbf{R_F}$ of the control varied from the standard value by more than ± 0.02 the paper was discarded.

$\mathbf{R_F}$ being defined as $A_M/(A_M + \alpha A_S)$, then

$$\alpha = \frac{A_M}{A_S}\left(\frac{1}{\mathbf{R_F}} - 1\right) = K\left(\frac{1}{\mathbf{R_F}} - 1\right)$$

If, as in Martin's analysis, the molecule of component 1 is substituted by d x-groups, e y-groups, etc., to form molecule 2, then comparing these two, in the same pair of solvents at the same temperature,

$$RT \ln \alpha_2 = \Delta\mu_1 + d\Delta\mu_x + e\Delta\mu_y \ldots$$

or

$$RT \ln K\left(\frac{1}{\mathbf{R_F}} - 1\right) = \Delta\mu_1 + d\Delta\mu_x + e\Delta\mu_y \ldots$$

This equation was tested by plotting log $[(1/\mathbf{R_F}) - 1]$ against d, while $\Delta\mu_1$, e, etc., remain constant. If the equation holds, a straight-line plot should be obtained. Tests, with different solvent mixtures, of a large number of derivatives led the authors to state that "The expectation of a close relationship between $\mathbf{R_F}$ value and chemical constitution, especially in respect to nature and number of these particular substituent groups, has been amply borne out."

Bate-Smith and Westall defined a term $\mathbf{R_M}$:

$$\mathbf{R_M} = \log\left(\frac{1}{\mathbf{R_F}} - 1\right)$$

which is a function of the temperature and the relative volumes of the solvent phases. They point out that if the relations suggested by Martin

are valid ΔR_M for a given substituent should be a constant. This was found to be so in a large number of instances. Thus glucosidation of position 5 of anthocyanidin monoglucosides to produce diglucosides yielded for ΔR_M in butanol–acetic–water (4:1:5, v/v/v): pelargonidin, 0.43; cyanidin, 0.42; peonidin, 0.40; malvidin, 0.38; and hirsutidin, 0.40. In other cases the constancy was not present. The authors found a tendency for ΔR_M of a particular type of substitution to decrease with increase in R_M, and suggested that "the change in chemical potential caused by a substituent group is less, the greater is the polar substitution already existing in the molecule." In a further study Bradfield and Bate-Smith (102) found that the introduction of a third hydroxyl group in a vicinal position in a benzene ring already substituted with two *ortho* hydroxyl groups in each case increased R_M, but the increase was not constant, and this indicated that other factors played a role.

The relationship between chemical constitution and R_F worked out by Martin is a useful and suggestive advance in a direction in which all separation techniques endeavor to move, namely, to derive from the behavior of the substances during separation firm information about their identity (298,555,792,817). It may also help in clarifying the mechanism of the separation. Thus as additional examples of the first kind of information, Jeanes *et al.* (448) detected an essentially linear relationship between R_M and the (assumed) degree of polymerization of a series of polymers of glucose obtained in the hydrolysis of potato amylose with malt α-amylase, and Nicholas and Rimington (689) reported an inverse linear relationship between R_F values of free porphyrins in the lutidine-water system and the number of carboxyl groups in the molecules. These authors used the value of the R_F of newly found porphyrins to predict the number of —COOH groups in the molecules. Also Bremner and Kenten (108) found that the linear relationship between R_M value and number of $—CH_2—$ groups in the molecule held well for an homologous series of primary *n*-alkyl monoamines and for a series of polymethylene diamines. Dedonder (210) has utilized a relationship between R_F and structure of heterosides to deduce the structure of a compound that gave a value for R_F different from that calculated for the accepted structure, suggesting that a change had occurred in the molecule owing to the pH of the developer.

As an example, perhaps, of the second kind of information, Isherwood and Jermyn (435) found a linear relationship between R_M and -log N, where N is the mole fraction of water in the developer solvent at 20°C. Developers prepared from 12 different organic substances were plotted in terms of their water content against the R_M's found for rhamnose, xylose, and glucose in these developers. The values for each sugar fell on its own straight line for all the developers except those containing phenol and *m*-

cresol. Here special hydroxyl interaction was suggested; in the other instances the heavy hydration of the sugar molecules seemed to play the determinative role in the distribution (see Fig. XIV-1).

VI. PIEROTTI AND CO-WORKERS' APPROACH

This work is perhaps at the point where it is directly applicable to chromatographic problems (733a,741a). Certainly, the method holds great promise. The authors start with essentially the same premises as Langmuir and Martin: premises basic to physical chemistry, of course.

The correlations derived by these authors (733) relate the activity coefficient at infinite dilution to the chemical structure of solute and solvent. The activity at infinite dilution was measured by appropriate means for a wide variety of solutes and solvents.

The correlations were set up on the following argument. The logarithm of the activity coefficient is a measure of the partial molal excess free

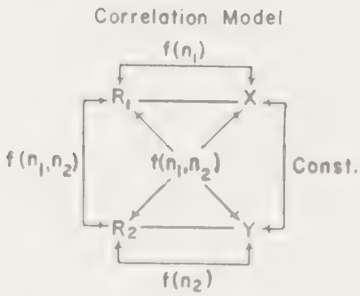


Fig. XIII-1. R_1 and R_2 are the alkyl groups of the solute and solvent molecules, respectively; x and y are functional groups such as —OH, —O—, —CO—, —Cl, etc.; and n_1 and n_2 are the numbers of carbon atoms in the respective R groups. The f's represent values of functions. (From Pierotti and co-workers (733).)

energy of solution of a substance, and this is the difference between the free energy of the substance in the pure liquid state (surrounded by molecules of its own kind) and the free energy it shows in the environment of the solution in question. This difference, the excess free energy, is related to the interactions (Chapter III) between the molecules in the pure liquid (standard) state and in the solution. It is assumed, as a first approximation, that the energies resulting from these forces are additive, and that they comprise the sum of the energies resulting from the forces which the various groups (—CH_2—, —OH, —O—, —Cl, etc.) in one molecule exert on the groups of surrounding molecules. The diagram shown in Fig. XIII-1 illustrates the interaction in question and defines some of the terms.

A few of the findings are as follows. With paraffins dissolved in paraffins

he relationship was quite simple (121): $\log \gamma^{\infty} = D(n_1 - n_2)^2$. The D onstant represents methylene interactions. When the data for ethanol, henol, or methyl ethyl ketone (MEK) dissolved in paraffin solvents from C[illegible] to C[illegible] were examined (Fig. XIII-2) it was found that the above D alue fitted in the relation $\log \gamma^{\infty} = K + D(n_1 - n_2)^2$. The K's were ifferent for the different functional groups (and temperatures) and epresent in part the interaction of these groups with the methylene groups f the paraffin solvents. The D's were evidently independent of the naure of the solute. When water was one of the components it was treated s a functional group only. A large number of compounds of different unctional types were studied as solutes in water. The data are shown in Table XIII-4. The forms of the relationships in the last column of the

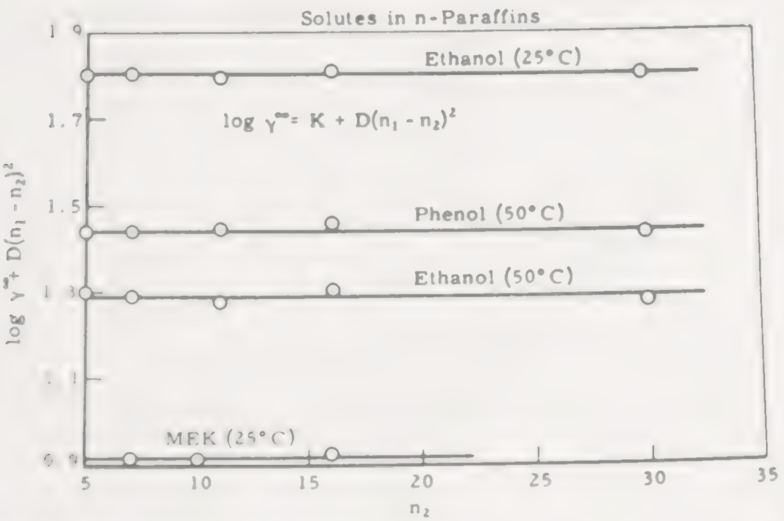


Fig. XIII-2. The correlation is shown between the data for ethanol (at 25° and 50°C.), phenol, and methyl ethyl ketone (MEK) dissolved in *n*-paraffin solvents of different chain lengths. For the meanings of the equation, see text. (From Pierotti *et al.* (733).)

table bring out further interesting points. The C term may be thought of as representing the dilution effect of CH_2 groups on the interactions of the functional group of the solute with the solvent. The B_{n_1} term may be thought of as representing the interaction of the CH_2 groups in the solute with the functional group of the solvent (water, in Table XIII-4). The n', n'' terms enter when secondary compounds, or ketones, are the molecules in question. This symmetry effect is necessary because in these cases (secondary alcohols, ketones, ethers, acetals, secondary amines) the functional group can be in different positions in the molecule. The n' and n'' are the numbers of carbons in the individual branches, as in MEK ketone.

TABLE XIII-4
Various Solutes in Water[a,b]

Solute	t, °C.	K	C	B	Equation $\log \gamma^{\infty}$ =
Normal acids	25	−1.000	0.490	0.622	$K + C/n_1 + B_{n_1}$
	50	−0.800	0.290	0.590	
	100	−0.620	0.140	0.517	
Primary alcohols	25	−0.995	0.558	0.622	$K + C/n_1 + B_{n_1}$
	60	−0.755	0.460	0.583	
	100	−0.420	0.230	0.517	
Normal secondary alcohols	25	−1.220	0.170	0.622	$K + C(1/n_1' + 1/n_1'') + B(n_1' + n_1'' - 1)$
	60	−1.023	0.252	0.583	
	100	−0.870	0.400	0.517	
Tertiary alcohols	25	−1.740	0.170	0.622	$K + C(1/n_1' + 1/n_1'' + 1/n_1''') + B(n_1' + n_1'' + n_1''' - 2)$
	60	−1.477	0.252	0.583	
	100	−1.291	0.400	0.517	
n-Allyl alcohols	25	−1.180	0.558	0.622	$K + C/n_1 + B(n_1)$
	60	−0.929	0.460	0.583	
	100	−0.650	0.230	0.517	
n-Aldehydes	25	−0.780	0.320	0.622	$K + C/n_1 + B(n_1)$
	60	−0.400	0.210	0.583	
	100	−0.03	0.0	0.517	
Acrolein	25	−0.720	0.320	0.622	$K + C/n_1 + B(n_1)$
	60	−0.540	0.210	0.583	
	100	−0.298	0.0	0.517	

Normal ketones	25	−1.475	0.500	0.622	$K + C(1/n_1' + 1/n_1'') + B(n_1' + n_1'' - 1)$
	60	−1.040	0.330	0.583	
	100	−0.621	0.200	0.517	
Acetals	25	−2.556	0.486	0.622	$K + C(1/n_1' + 1/n_1'' + 2/n_1''') + B(n_1' + n_1'' + n_1''')$
	60	−2.184	0.451	0.583	
	100	−1.780	0.426	0.517	
n-Ethers	20	−0.770	0.195	0.640	$K + C(1/n_1' + 1/n_1'') + B(n_1' + n_1'')$
n-Nitriles	25	−0.587	0.760	0.622	$K + C/n_1 + B(n_1)$
	60	−0.368	0.413	0.583	
	100	−0.095	0.00	0.517	
n-Acrylonitriles	25	−0.520	0.760	0.622	$K + C/n_1 + B(n_1)$
	60	−0.323	0.413	0.583	
	100	−0.074	0.00	0.517	
Normal esters[c]	20	−0.930	0.260	0.640	$K + C(1/n_1' + 1/n_1'') + B(n_1' + n_1'')$
Normal formate esters	20	−0.585	0.260	0.640	$K + C(1/n_1' + 1/n_1'') + B(n_1' + n_1'')$
Normal mono alkyl chlorides	20	1.265	+0.073	0.640	$K + C/n_1 + Bn_1$
n-Paraffins	16	0.688	—	0.642	$K + Bn_1$
n-Alkyl benzenes	25	3.554	−0.466	0.622	$K + C/n_1 + Bn_1$

[a] From Pierotti *et al.* (733).
[b] For the meanings of C, B, n_1, n_2, n', n'', n''' see text.
[c] Does not include formate esters.

$n' = 1$, $n'' = 2$. In the case of tertiary compounds, the third branch is accounted for by n'''. These data are only a small extract from the information worked out by Pierotti and his group.

The application of these correlations to distribution of a solute between two phases follows from the fact that the distribution ratio of the solute is the inverse ratio of its activity coefficients in the two phases. As Pierotti and co-workers point out, the γ^{∞}'s present a means of calculating distribution relations for an homologous series between a solvent pair. Calculations indicate that the resulting equation has the form:

$$\text{log distribution ratio} = k + bn$$

This states that the log of the distribution ratio of homologous solutes in a given pair of solvents varies linearly with the number of carbon atoms

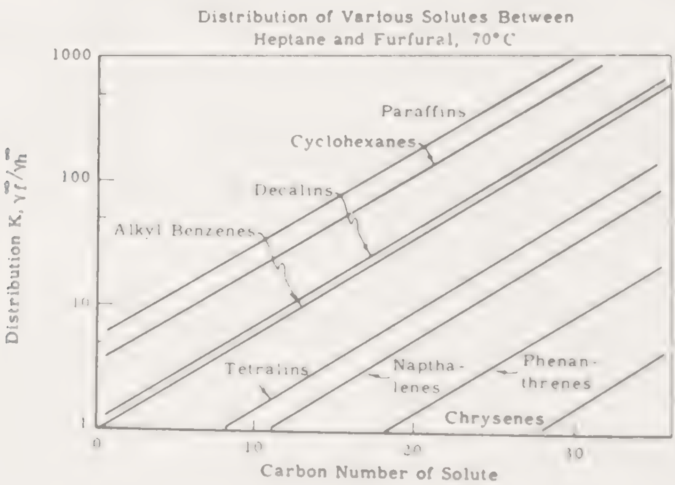


Fig. XIII-3. Distribution of various solutes between heptane and furfural at 70°C. (From Pierotti and co-workers (733).)

This, of course, is a general statement of which Traube's rule is a special case.

A few data are plotted in Fig. XIII-3 to show the scope of these relationships. The figure shows the distribution ratios of various kinds of hydrocarbons between heptane and furfural at 70°C, plotted against number of carbon atoms in the hydrocarbon solute. How well any two types can be separated by this solvent pair can be inferred from the vertical distance between the appropriate lines, which correspond to the ratio of K values (distribution values). The effect of molecular weight on the extraction can be seen also. The authors point out that the

horizontal distance between the appropriate parallel lines represents the maximum spread of carbon numbers compatible with separation.

It seems likely that as investigations of the kinds reported in this chapter are pressed the practice of chromatography will become much more rational than it has been.

On Choosing Mobile and Stationary Phases (Solvents, Developers, Adsorbents)

I. INTRODUCTION

As has been repeatedly emphasized, the key phenomenon in chromatography is the distribution of the mixture to be separated between a mobile and a stationary phase. The mixture is given. Sometimes it is possible by modifying it to improve our chances for separating it. The pair of phases we have to choose. This choice will generally be aided by consultation of the literature. However, since we wish to deal with the choice of phases in the most complete and general way, we will not rest content with this valuable aid alone.

In this chapter we will discuss chiefly the choice of phases for column and paper chromatography. In Chapter VIII, Section VII, the special problems of adsorption chromatography were summarized. The general principles advanced in this chapter for choosing phases in connection with liquid–liquid partition can be extended to adsorption partition via the special considerations discussed more appropriately in Chapter VIII.

II. PRELIMINARY RULES

In liquid–liquid chromatography our objective is to influence R or R_F. It was pointed out in Chapter XIII that, *in general, we can influence* R *or* R_F *more effectively through* α *than through* A_M *or* A_S. This rule applies to all kinds of chromatography.

From the theoretical definition of R and R_F it is evident that *if a solute is driven into the stationary phase* α *will be increased, and* R *or* R_F *will decrease.* This is another way of saying that the zone of the substance will be retarded. This is what is observed.

In the conventional partition chromatogram, whether columns, paper strips or sheets are used, the stationary phase will be more polar than the mobile phase since it must wet and adhere to a polar support. We can see then, that under these circumstances, *the zone of a substance which is preferentially distributed into the more polar phase will have the lower* R *or* R_F *value.*

On the basis of these considerations we can also say that normally in all these systems *the lower the* R *or* R_F *value, the more polar the substance of the zone.*

All these rules are contingent; however, they work sufficiently well for most purposes. Furthermore, if the "reversed phase" partition chromatography is used, where the stationary phase is less polar, then we must suitably reverse these rules.

III. PROCEDURAL STEPS

The first step in choosing a solvent system is to be sure that the substances can be recognized when they are separated. That is to say, it must be possible to recognize zones when they are formed (Chapter XII).

The second step is to consult the literature in the form of the numerous compilations of data that are available (84,362,529,709,888,1033,1035). This may solve the problem. But it may be desired to improve a method or to deal with some new compound or compounds that have appeared in the course of following a previously satisfactory technique.

The third step, then, is to consider the circumstances under which the mixture was obtained (see Chapter XV). First is it hydrophilic or lipophilic; then, what is its likely nature?

Hydrophilic substances are likely to be rather polar. They may or may not ionize in water. If they ionize they may be basic, acidic, or amphoteric.

Lipophilic substances are generally relatively nonpolar. They are more soluble in the less polar organic solvents than in water and the more polar solvents, though not invariably so when in mixtures. The solubilizing behavior of other components may obscure the picture.

Examples of ways of controlling **R** or $\mathbf{R_F}$ values of these different types of substances will, together with discussions based on the principles in Chapter III, serve to explain the choice of mobile and stationary phases.

The fourth step is to try out the system on a small scale. This can usually be done in a relatively short time by using a small-column technique (163,167) or the Rockland and Dunn method (787) with small paper strips or sheets. This step is essential because of the contingency of the rule of procedure. At this stage, the solvent systems can be modified in the light of steps 2 and 3, and of the results of step 4, thus leading to the best solution of the particular problem. *The approach should above all be reasoned and systematic.*

A point worth emphasizing has been made by Hais (362), namely, that certain developers may have to be avoided because they disturb or prevent the detection of zones. He gives as an example phenol, which if it were used in a developer would have to be very carefully removed from the paper or zone material later (by heating, aeration, ether or toluene extraction) if it were intended to look for zones with a diazonium reagent.

or with the use of ultraviolet absorption, or possibly fluorescence. Also, since it is toxic to microorganisms, its presence would hinder biological assay.

IV. EXAMPLES OF PROCEDURES

Examples from the literature will be used to show the rationale behind choices of phases. These examples will be chosen to cover lipophilic and less lipophilic substances, and hydrophilic substances of different types. The objective will be to find a pattern that can be generally applied.

1. Steroids

Steroids are lipophilic substances concerning the chromatography of which a considerable literature has accumulated. Savard (811) has given a bibliography. He noted that twelve developer systems had been described for the paper chromatography of ketosteroids. These generally comprised a relatively polar, nonvolatile stationary phase, such as propylene glycol, formamide, nitromethane, Phenyl Cellosolve, or Carbitol, and a nonpolar, volatile mobile phase, such as benzene, toluene, ligroin (60° to 90°C.), petroleum ether. (Reversed phase techniques have been developed, but will not concern us here.) In each case, of course, the stationary and mobile phases are equilibrated and thus are mixtures, but for the sake of simplicity the major component alone will be used to designate the phase. Thus a "propylene glycol" stationary phase used with a "benzene" mobile phase will contain benzene, as will the mobile phase contain propylene glycol.

It was observed that when benzene or toluene comprised the mobile phase the less oxygenated (less polar) steroids traveled close to the front and were poorly resolved. They could be driven more into the polar stationary phase by using a poorer (even less polar) solvent such as ligroin, whereupon satisfactory retardation with accompanying resolution was achieved. Alternatively, satisfactory retardation could be achieved by improving the dissolving power of the stationary phase for these less polar steroids. This was accomplished by using Neher and Wettstein's (681) stationary phase, ethylene glycol monophenyl ether (Phenyl Cellosolve).

Thus, according to this rationale, a set of three solvent systems could be used to separate groups of steroids. In general, the most polar steroids (oxygen to carbon ratio high) were best separated among themselves from each other by a toluene–propylene glycol system; the less oxygenated ones by a ligroin–propylene glycol system; and the least polar ones, with no —OH group, only 1 or 2 keto groups and a content of 19 carbons or more, with a ligroin–Phenyl Cellosolve system. When the first two

groups are present together, the ligroin–propylene glycol system is used even though the very oxygenated steroids move extremely slowly in it.

Savard uses an R_T value for characterizing the steroid zones (see Chapter VII, Section X). The value represents the mobility of the zone in terms of a reference standard. The higher the R_T value, the higher the mobility; the lower the R_T, the greater the retardation of the zone of steroid. To compare, then, for a given mobile-stationary phase system, a group of β-ketosteroids (Table XIV-1), it is quite evident that the higher the oxygen

TABLE XIV-1

Relative Mobilities (R_T) of β-Ketosteroids in Ligroin–Propylene Glycol[a]

Class No.	Compounds	No. of C Atoms	No. of C=O	No. of —OH	R_T
V	Δ[5]-Androstene-3β,14α-diol-17-one	19	1	2	0.034
V	Pregnane-3β,17α-diol-20-one	21	1	2	0.21
V	Δ[5]-Pregnene-3β,21-diol-20-one	21	1	2	0.34
III	Androstane-3β-ol-16-one	19	1	1	0.50–0.70
III	Dehydroepiandrosterone	19	1	1	0.70
III	Epiandrosterone	19	1	1	
III	Etiocholan-3β-ol-17-one	19	1	1	1.00
III	Δ[5]-Pregnen-3β-ol-20-one	21	1	1	1.20
III	Allopregnan-3β-20-one	21	1	1	1.20

[a] From Savard (811).

to carbon ratio the greater the retardation, and with a given ratio the more —OH groups present the greater the retardation. These data speak for a competition between dipole interaction plus hydrogen bridging in bringing the molecule into the propylene glycol phase, and London dispersion forces in making it more soluble in the ligroin phase. This gives the rationale for the choice of solvents when lipophilic molecules with polar groups are to be separated. The operation of induction interactions which tend to bring the unsaturated steroid more into the stationary phase than the saturated and so retard these steroids is shown in Table XIV-2 and the increased effects in this respect of multiple unsaturation and conjugation are shown in Fig. VII-15.

However, the situation is not quite so straight-forward as the discussion so far might imply, and Savard has indicated this. There exist subtle factors which seem to depend on the geometry of the molecule. Thus two or more oxygen functions exhibit less retarding effect than predicted by the simple statements above when they are adjacent (a phenomenon noticed also with sugars and other substances, see below). Presumably an internal depolarization occurs which disposes these molecules relatively more

TABLE XIV-2

Effect of Conjugated Double Bond on Mobility of Ketosteroids[a]

Saturated Steroids	R_T	Unsaturated Steroids	R_T
Pregnane-3,20-dione	3.0	Progesterone	2.3
Androstane-3,17-dione	2.5	Δ^4-Androstene-3,17-dione	1.7
Progesterone	2.3	Δ^{16}-Progesterone	2.1
Allopregnane-21-ol-3,20-dione	1.06	Deoxycorticosterone	0.45
Androstane-11β-ol-3,17-dione	0.17	Δ^4-Androstene-11β-ol-3,17-dione	0.10

[a] From Savard (811).

toward the nonpolar mobile phase. And again, the conformation of the steroid molecule seems to be such (40–42) that equatorially oriented substituents, such as hydroxyls, are less hindered, and so more able to interact with solvent, than axially oriented substituents, and this is borne out almost without exception by the greater retardation of the former than the latter of the two epimeric forms (811).

Such fine distinctions, which are evident usually *ex post facto*, serve to increase our confidence in the grosser reasoning, and when based on such careful quantitative studies as Savard has published they greatly aid identification of the substances and suggest structural possibilities for unknowns. For the initial choice of phases we usually must rely on the reasoning based on broader polarity concepts. Then, following such precedents as this, and reasoning by analogy, the choice of solvent systems can be progressively refined.

2. Higher Fatty Acids

Higher fatty acids are lipophilic substances, but consideration of them will be given below under fatty acids that are water-soluble (Section IV, 5), since the extension from one class of solubility behavior to the other is instructive.

3. Pattern for Lipophilic Substances

In general we can say concerning lipophilic materials that in ordinary paper or column partition chromatography, one of the phases used should be somewhat polar, so that it will interact firmly with the support and not be extremely soluble in the other, mobile phase. The latter should be essentially nonpolar.

If the mixture is overly retarded, indicating that it has polar characteristics, it can be made more mobile either by decreasing the polarity of the supported phase, or, usually better, by increasing the polarity of the

mobile phase. These changes can be made by changing the phases to other chemical species or by admixture of appropriate agents.

If the mixture runs too fast (high R_F's and unresolved zones), indicating nonpolar characteristics, it can be retarded by making the supported phase somewhat less polar.

If the mixture moves along the paper or column but is not well resolved it may be necessary to try the effects of small changes in the structure of the phases: for example, to change from hexane to octane or isooctane or to cyclohexane, or methyl cyclohexane for mobile phases; or benzene to toluene, to xylenes, the order of increasing basicity (363,404,579), or electron-donor capacity, and thus in this respect "polarity," and solvent power (401). Here it may be a matter of running many small-scale experiments until some recognizable pattern of behavior emerges.

If none of these expedients works, then it may suffice to use a very low or relatively low R_F system with prolonged development.

4. Neutral Hydrophilic Substances

There are a great many *hydrophilic substances* that are neutral: lower alcohols, polyhydric compounds, lower carbonyl compounds, and so on. We shall take the paper chromatographic separation of *sugars* as an example from which to draw the proposals that can be applied to other neutral hydrophilic substances. In this area a great deal of data are drawn together by Kowkabany (493) and by Binkley (75).

The very strong effect of water in any developer used with sugars is certainly to be expected, since the great solubility of most sugars in water and the strong hydration of these substances would suggest it. Jermyn and Isherwood (435,452) have given precision to this relationship by plotting log $[(1/R_F) - 1]$ against the negative log of the mole fraction of water ($-\log N$) for a group of solvents saturated with water at 20°C., for three representative sugars. The data are shown in Fig. XIV-1. These data (phenol and *m*-cresol are out of line) illustrate that with such neutral hydrophilic substances as the sugars the water content of the mobile phase can have an important effect on the R_F value. The relation found by Jermyn and Isherwood is essentially that as the quantity of saturation water is decreased in the mobile phase by choosing a less hydrophilic organic solvent, the R_F value goes down. Thus different solvents (except phenols) may be compared on this basis. Their quantitative statement of the relation makes quantitative comparison possible with these binary mixtures, and suggests a method for controlling R_F values by changing from one solvent to another of different polarity and water-holding capacity. With phenolic solvents the sugars ran faster than would be expected on

the basis of water content (Fig. XIV-1). This seemed to indicate that the phenols might be bound to the sugars, depolarizing them in some way.

A more detailed examination of the R_F value as it is correlated with the structure and configuration of the sugars, using as the solvent ethyl acetate-pyridine-water (2:1:2 by volume, using the water-poor phase), indicated the following. The R_F values were less sensitive to numbers of —OH groups in the molecules than to the configurations of the rings, and when rings of the same size and with the same configuration were compared the substituent on C_5 or on C_1 determined the R_F value strongly. Thus, specifically, if an aldomethylose is compared with the corresponding

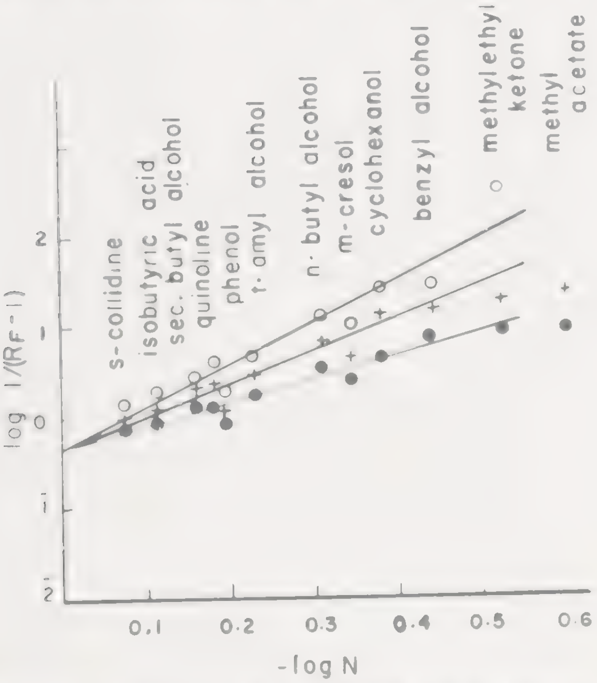


Fig. XIV-1. Relationship between R_F value and molar fraction of water in non-aqueous phase. ○, rhamnose; +, xylose; ●, glucose. Redrawn from Isherwood and Jermyn (435).

aldohexose and aldoheptose (the terminal groups being CH_3, CH_2OH, and $CHOHCH_2OH$, respectively) the R_F observed for the methylose is about 50% higher, and that for the heptose 15% lower than the corresponding aldohexose. This is the order of increasing polarity of the substituent. When an aldopentose is compared with the corresponding keto hexose, which differs by having $-CH_2OH$ in place of $-H$ on carbon 1, the ketohexose R_F value is some 10% below that of the aldopentose, again showing the effect of increased polarity in retarding the sugar. When the effects of the configuration of vicinal —OH groups are examined it appears in some

cases that where a pair of sugars differ only in the configurations of two neighboring—OH groups, if the groups are both on one side of the ring (the *cis* arrangement) the R_F will be higher than if they are *trans*. This implies a depolarizing interaction such as we have noticed before with sterols.

The situation is by no means quite simple and straightforward, and Isherwood and Jermyn have pointed out, among the factors that make clarification difficult, the complex interrelations between aldose (or ketose) and furanose and pyranose forms that may exist in a solution. Sugars set in the furanose forms by methyl glycoside formation always showed considerably higher R_F values than the corresponding methyl glycosides in the pyranose form.

One regularity observed by these investigators is that among the aldopentoses and aldohexoses the R_F values are on the whole inversely proportional to the melting points. They suggest that the melting point measures an interacting ability between the molecules in the crystal which reasonably correlates with the affinity of these hydroxylated molecules for water. It would be reasonable to suppose that we have here another manifestation of a fairly widely applicable correlation between melting point (of polar substances) and solubility in water: the higher the melting point of the substance, the more soluble it is likely to be in a polar solvent. Of course, reasonably similar compounds must be compared.

One additional evidence of interactions may be mentioned. French and Wild (298) have shown a relation with diagnostic and predictive value between R_F value (or a derived function) and molecular size and structure of many oligosaccharides. A distribution parameter α' is defined as $\alpha' = R_F/(1 - R_F)$. Then, using Martin's relation that connects R_F with the constitution of a substance (Chapter XIII, Section V) it is shown that $\log \alpha' = C_1 - nC_2 - mC_3 \ldots$. Here "$C_1$ is the value of $\log \alpha'$ for the first member of a saccharide series and the other constants C_2, C_3, etc., are characteristics for the monosaccharide unit being added (glucose, galactose, arabinose, etc.), its position of attachment, ring form, anomeric configuration, etc." Using this relation these investigators have shown "that the logarithm of the partition function α' is an additive property of the various structural features of a given oligosaccharide molecule, and that given suitable reference compounds one may predict with reasonable accuracy the papergram mobility for a saccharide of any given structure. Conversely, the papergram mobility may be a very useful indication as to possible structural features of incompletely known saccharides, and thus facilitate the more rigorous structure analysis." The solvent system used was butanol : pyridine : water 6 : 4 : 3 by volume. Eaton and Dickeman paper No. 613 was used. Evidently in all these

cases increasing the molecular weight and hydrophilic nature of the substance lowered the R_F. (In some cases the R_F was so low that the solvent had to be passed over the paper fifteen or more times, giving the effect of a very prolonged development, before adequate resolution was obtained.)

In choosing the best solvent mixture for separating mono- and oligosaccharides Jeanes, Wise, and Dimler (448) have set down certain procedures that would appear to have general applicability for all sorts of mixtures. They have studied the separations of sugars using some 16 developer mixtures, with one passage of the developer in each case. For each developer they calculated the ratio (R_F maltose/R_F glucose) $\times$ 100. This gave a picture of the separating ability of these solvents for the test pair of sugars. As an adjunct to this information another parameter was devised, namely, the number of developments (in which the paper was dried and redeveloped with the same developer in the same direction so as to give increased effective distance of travel of solvent) needed to give optimum resolution of low-R_F spots.

These authors then recommend that the first step with a new mixture should be a preliminary chromatogram of the mixture with a developer that separates maltose and glucose moderately well. This corresponds with our fourth step in Section III. They suggest 1-butanol–pyridine–water (3:2:1.5—by volume before separation of the organic phase; ratio value 0.64), since it separates moderately fast-running sugars and is likely to give useful information about slower, and faster, sugars. This preliminary chromatogram gives an indication of the complexity of the initial mixture, and of the rate of movement of the various components. Then for a mixture which by this test is largely composed of low-R_F components a developer with a high R_F maltose/R_F glucose would be taken. This is because not only do the zones of sugars move more rapidly in such developers, but also the resolving effect on them is greater. The resolving power reaches a maximum as the R_F maltose/R_F glucose ratio is increased and this maximum comes at higher values of the ratio for lower-R_F sugars. Thus for the slower sugars (disaccharides and other oligosaccharides, for example) a ratio of about 0.8 is desirable, such as given by fusel oil (121 to 129°C.)–pyridine–water, 1:1:1, ratio value 0.83. For such a separation multiple or continued development may be desirable, and the number of developments for maximum resolution n is given by the relation $n = (1/R_F) - 1$. Where with this solvent an R_F of 0.29 is observed for cellobiose, then the best number of developments to separate sugars of this general mobility will be 2 to 3. It will of course be a larger number for slower sugars. For faster sugars that behave like pentose, a developer of ratio about 0.54 is recommended, namely 1-butanol–pyridine–water (3:1:1.5 by volume before separating the organic phase). Here not more than 2 de-

velopments would be worth while. In general pentoses ran faster than hexoses, and mono- than disaccharides. Also in an homologous series, the R_F decreased with molecular weight, and was higher in 1,4-linked disaccharides than in the corresponding 1,6-linked ones.

For a summary of the effects of solvents the authors' work must be consulted. Ternary solvents composed of an alcohol (1-butanol or fusel oil), a base (pyridine or symmetrical collidine), and water were used. With the base and water the same, and the same volume ratios (before separating the organic phase) much lower R_F's were obtained with fusel oil than with 1-butanol. Otherwise identical mixtures gave much lower R_F values when collidine replaced pyridine. Increase in the proportion of pyridine in the mixture increased R_F values, as did increase in water content. The direction of these changes is consistent with the interpretation that by increasing the ratio of polar components in the mobile phase the R_F's of the carbohydrates are increased. This gives the rationale for adjusting the developer mixture in the necessary direction on the basis of the results of the recommended preliminary chromatogram.

5. Acidic Hydrophilic Substances

As examples of *hydrophilic* substances that are *acidic*, we can take two types: the volatile aliphatic acids (giving some attention also to the less hydrophilic, higher acids) and the nonvolatile dicarboxylic acids.

The problem essentially is to drive these quite polar substances sufficiently out of the polar stationary phases into the less polar mobile phases of the ordinary paper or column partition methods. Success is attained by suppressing the ionization of the nonvolatile acids, by the use of a relatively strong acid in the mobile phase, playing also on any differences in ionization constant to help distinguish the acids in the mixture from each other. Volatile acids are converted to nonvolatile derivatives when paper chromatography is the method for separation (123,285, 411,467). When column partition chromatography is used, the volatile lower acids can be used directly, since the walls of the tube retain them (264).

The separation *of volatile acids* on paper serves as a first example (467). The acids, applied to the paper as ammonium salts, were developed with ammoniacal alcohol (100 ml. 95% ethyl alcohol plus 1 ml. conc. ammonium hydroxide). Other solvent systems could be used, and it was possible markedly to change the R_F values by changing the per cent water present. The R_F values are shown in Table XIV.3. The increase in R_F with increase in chain length correlates with the supposition that the mobile phase is less polar than the stationary. Fitting in, also, for reasons already dis-

TABLE XIV-3
R_F Values of Acids Developed with Ammoniacal Ethyl Alcohol Solution[a]

Acid	R_F	Acid	R_F
Formic	0.31	*n*-Caproic	0.68
Acetic	0.33	1-Heptanoic	0.72
Propionic	0.44	1-Octanoic	0.76
n-Butyric	0.54	Vinyl acetic	0.46
n-Valeric	0.60	*n*-β-ketocaproic	0.53

[a] From Kennedy and Barker (467).

cussed above in Chapter III, is the observed lowering of R_F of vinylacetic as compared with butyric because of the presence of the polarizable double bond. The lowered R_F of the keto caproic compared with caproic is here attributable to the keto group and the active, acidic α-hydrogen.

The separation of *fatty acids* in partition columns presents an interesting example because of the wide range of polarities covered from acetic to stearic. The example is taken from Ramsey and Patterson's work (753). They separated acetic, propionic, and butyric acids on a silicic acid column, the stationary phase of which was aqueous, and the mobile, 1% butanol in chloroform washed free from alcohol and equilibrated with water. The butyric acid zone showed the highest R_F in this relatively nonpolar developer. When it had emerged, the developer was changed to 10% butanol in unwashed chloroform, equilibrated with water, whereupon the propionic and acetic zones were speeded up and emerged in that order.

To separate the next higher acids, C_5 to C_{10}, a less polar stationary phase was needed (755). These acids would have moved with or just ahead of butyric in the water–chloroform–butanol system, with little or no differentiation, being themselves nonpolar. The system found useful here was a stationary phase of methanol, supported on silicic acid, and a mobile phase of isooctane. Again, the acids were separated with the C_{10} passing out of the column first, followed in turn with the acids of decreasing carbon number, the C_5 acid appearing last.

Finally, these investigators (756) were able to separate the still less polar acids C_{11} to C_{18} by using a slightly basic stationary phase composed of furfuryl alcohol mixed with 2-aminopyridine, and a mobile phase of n-hexane, which is a rather poor solvent for the higher fatty acids. Again, the higher acids showed the higher R_F values.

The rationale here clearly follows the type of pattern outlined in Section IV.3 above. Retardation in each case depends on bringing the lower homologs of the group more into the stationary phase than the higher. This plays on ionization in water, for C_2 to C_4 acids, combined with competition for the acid from the butanol and the chloroform in the mobile

phase. The side chain of the C_5 to C_{10} group is too effective in bringin these acids into the mobile phase, whereas their solubility in water too low to aid retardation. For these reasons a better solvent must be us as the stationary phase, and a poorer solvent for the mobile phase. (Th almost follows, since the two phases must not be too miscible.) Lastly, the C_{11} to C_{19} range, where the acids are almost nonpolar substances, the have to be drawn into the stationary phase by the aid of base, while th mobile phase separates them (very likely) through dispersion interaction which increase in total effectiveness with increase in number of carbons the side chain.

The separation of *dicarboxylic acids*, which are nonvolatile, strong aci presents interesting requirements for the choice of phases. The sep ration on paper will be discussed briefly, and arbitrarily, since the larg literature cannot be reviewed (84). (Block, Durrum, and Zweig list 2 solvent systems, as some of those that have been used.) What the prob lem amounts to essentially is this. If di- and tricarboxylic acids and hy droxy and keto derivatives are applied to paper and developed with wate they will in general show high R_F values and little separation, many of the moving at the front of the developer. On the other hand, as they are no very soluble in many nonpolar solvents, if development were carried ou with any of these, the R_F values would be extremely low, the acids tendin to remain in the aqueous stationary phase on the paper. If, however, "swamping acid" is added in considerable excess (compared to the concen tration of polycarboxylic acids) they will be solubilized in an organic mobil phase, and so a separation can be achieved. Formic is usually used a the swamping acid because it can be steamed out of the paper later. (The if the paper is sprayed with an indicator such as a 95% alcoholic, pH solution of bromophenol blue containing a trace of methyl yellow (367 the acid zones will stand out yellow on a blue background, and the zone of the stronger acids will have reddish center parts.)

The formic acid is not so strong an acid as some of the dicarboxylic acid (first ionization constants). Its ionization constant is 2×10^{-4}, com pared with 3.8×10^{-2} for oxalic; 1.6×10^{-3} for malonic; 1.1×10^{-3} fo tartaric. But it is stronger than others, for example, than succinic, $6.6 \times$ 10^{-5}; glutaric, 4.7×10^{-5}, and the higher dicarboxylic acids. It is clos to still others: citric, 8×10^{-4}; malic, 4×10^{-4}. The medium strengt of formic acid compared with the other acids probably partly explains it success as a swamping acid. As an example of data we take the repor by Buch, Montgomery, and Porter (132). The mobile phase was th organic layer from 1-pentanol-5*M* formic acid (1:1, v/v), prepared 3 o more hr beforehand. Some of the R_F values observed are shown in Tabl XIV-4.

TABLE XIV-4
R_F Values of Some Acids and Ionization Constants

Acid	$100 \times R_F$[a]	Dissociation constant[b] (First)
Oxalic	15	3.8×10^{-2}
Malonic	53	1.6×10^{-3}
Succinic	61	6.6×10^{-5}
Glutaric	78	4.7×10^{-5}
Adipic	75	3.7×10^{-5}
Azelaic	86	[c]
Sebacic	89	[c]
Malic	32	4×10^{-4}
Tartaric	14	1×10^{-3}
Ketoglutaric	48	
Glutamic	5	
α,β-Dihydroxybutyric	40	
α,γ-Dihydroxybutyric	29	

[a] From Buch, Montgomery, and Porter (132).
[b] From Lange (518).
[c] Probably of same order of magnitude as adipic.

It would appear that the effect of formic acid described above is borne out, not only for the unsubstituted dicarboxylic acids but also for malic and tartaric as compared with succinic. The hydroxyl group in malic and the two in tartaric undoubtedly contribute to the retardation of these acids. The keto group of ketoglutaric would be expected to contribute to retardation, and, in the case of glutamic, the great effect of the salt-forming amino group is apparent. The depolarizing effect of two neighboring hydroxyls can be observed reflected in the higher R_F of α,β-dihydroxy butyric acid compared to the α,γ-compound. Undoubtedly these interpretations are oversimplified, but they present throughout a consistent pattern for the choice of phases.

The pattern for basic substances follows that for acid, it would seem, with appropriate changes. Thus the swamping substance would be a base of appropriate strength, and weakly acidic solutes would be retarded more than neutral or basic ones, and so on.

6. Inorganic Substances

The choice of phases for separating inorganic cations has been discussed by Pollard and McOmie (739).

In the case of paper chromatography, where an organic mobile phase is used, the free cations or anions themselves will not be very soluble (along with their opposite ions) in the ionized form. Therefore, the procedure would seem to be, in principle, to choose a system which by complexa-

tion makes the cation (for example) more soluble in the mobile phase. This can often be done with strong hydrochloric acid, since the chloride ion in strong acid seems to have the ability to complex a very wide range of cations. Other complexing and chelating agents can be used, and Pollard and McOmie have prepared extensive tables of solvent systems that incorporate these agents.

With ion exchangers the principle seems to depend on charge or hydrated radius of the ions to make them sufficiently different that they can be separated on the column. With difficulty separable ions, as described in Chapter IX, Section X, in the separation of mercury from gold, complexation solves the problem. Citrate seems to be a useful complexing agent, able to convert plus charged ions to neutral or negatively charged particles, thus drastically changing their exchange behaviors. For example (828), Pr^{+3} may be complexed to $Pr(H_2Citrate)_3$; Y^{+3} to $Y(H_2Citrate)_3$; Sr^{+2} to $Sr(Citrate)^-$; and Ba^{+2} to $Ba(Citrate)^-$. These complexes form to various ratios at different pH's and this plus the relative concentration of citrate gives considerable control over **R** value. And, of course, the wide and increasing variety of complexing agents enriches the armamentarium of the chromatographer.

V. CONCLUDING REMARKS

The sets of procedures given above, along with interpretations, are designed to help the chromatographer derive patterns of procedure for choosing mobile and stationary phases for his own work. Only a little of this important and fascinating part of chromatography has been exposed. As with the approaches discussed in Chapter XIII this kind of heuristic will, as it is developed, help to widen the rational basis of chromatography.

As one looks at a long list of the developers that have been used for making a given separation, one is struck by the variety of substances that have been used. The first impression may be one of bewilderment. But as the names are replaced by structures of the molecules, and these by classes, a pattern very soon appears, couched, it is true, in terms like polar and nonpolar, and in somewhat more precise terms like strong acid, weak acid, neutral substance, weak base, strong base. This pattern, the heuristic or "method of the method" which we have developed in a rudimentary way in this chapter and have tried to suggest throughout the book, is a point of departure. From this point, a rational return can be made to the variety of reported systems to estimate the subtler influences through which one system serves better some particular separation than another system.

On Using Chromatography

I. INTRODUCTION

This chapter is designed to fortify the reader for that most difficult step: reduction to practice. It is not difficult to use chromatography to separate mixtures provided that one is able to mobilize general chemical knowledge and bring it to bear on the problem.

It may be pointed out that chromatography is an excellent channel through which to teach chemical principles. The concepts of equilibrium, steady state, nonequilibrium, the laws of solutions, of kinetics, the principle of Le Chatelier, all can be illustrated graphically. Many valuable chemical insights can be gained through exercises in interpreting the R_F values determined by others (Chapter XIV), in looking for correlations (Chapter XIII), or in choosing appropriate phases for a separation.

Another use of chromatography, though one more closely related in interest to the separation of mixtures, is for characterizing complex and unknown mixtures. This has had some forensic value. For example, Valentin was able to differentiate between natural and artificial Peru balsam because the natural product gave a much more complicated chromatographic *pattern* than the artificial substance when alcoholic extracts were chromatographed on alumina (955).

II. PRELIMINARY CONSIDERATIONS

Before beginning the chromatography of a mixture it is advisable to be sure that the mixture does indeed contain the substance being sought. This elementary point is occasionally overlooked, and chromatography (though unpublished) is done on a mixture that turns out to be empty of the desired product because this was destroyed in preliminary manipulations, or was not soluble in the solvent system used, or in some way escaped the operator.

There are, of course, those situations where no clearly known substance is being sought, but just "what's there." Indeed, chromatography opens to the synthetic organic chemist an attractive way of investigating the tars that are so often discarded—of, in fact, accounting for *all* the products of a reaction.

In general, in starting with complex or chemically inhomogeneous mixtures, it is most profitable to precede chromatography by chemical fractionation—as when one starts with an animal or plant tissue. A great deal has been written about such fractionations, but it is mostly scattered it seems. Some examples are presented here to indicate what may have to be done *before* chromatography can begin. But the treatment here is rudimentary. For further discussion and for methods of dealing with and identifying small amounts of products, see Cheronis (176a).

III. PRECHROMATOGRAPHY STEPS

Chromatography is applicable only to molecular mixtures. Thus if one wishes to isolate pigments from a leaf or fruit or sterols from an animal or plant tissue, or to analyze a tar or a mineral or the results of a synthetic or other reaction, the substances of interest in the tissue, mineral, or whatnot, must be brought into solution. This step is one of the prechromatography steps.

Another step is the physical or chemical fractionation that normally should precede chromatography of complicated mixtures. This step simplifies the problem in two ways: it simplifies the mixture, and it gives information needed for choosing the kind of chromatographic system to use and for making the choice of phases.

After these two steps, which we will term *A* and *B*, the chromatographer will have in hand solutions of various types which are to be chromatographed. These he proceeds with as will be indicated below.

IV. SYSTEMATIC SCHEMES

Two of these schemes are presented in outline form. They are rearranged somewhat to suit the convenience of this presentation. The first was kindly called to my attention by Dr. William A. Bulen.

1. Plant Tissue

The tissue (cells, leaves, etc.) is freeze-dried and the dried material is extracted with ether. (Lipoidal components of tissues may be bound to non-ether-soluble materials by bonds that are not broken by ether, but that are broken by more polar reagents like water or alcohol. A lookout should therefore be kept for the results of phenomena of this kind, which can often be utilized in making fine distinctions between classes of substances.) The tissue, treated in the above way, yields a residue and an ether extract. These may be handled as shown in the Tables XV-1 and XV-2, taken from Aronoff, Benson, Hassid, and Calvin (19). Each worker

TABLE XV-1

Treatment of a Residue from the Ether Extraction of Freeze-Dried Plant Tissue (19)

- Residue
  - Extraction with 80% EtOH-H_2O
    - Residue
      - Water extraction
        - Residue
          - Ethanolamine extraction
            - Residue
            - *Cellulose*
        - *Proteins*, etc.
          - Phosphotungstate
            - *Proteins*
            - *Supernatant*
    - *Sugars, organic acids, amino acids*
      Ethanol is removed and a cation exchanger is employed (Duolite C-3)
      - Retarded by exchanger: amino acids
        - H^+ → *Amino acids*
      - Sugars & organic acids
        - Anion exchanger used (Duolite A-3)
          - Retarded by exchanger: Organic acids
            - OH^- → *Organic acids*, as salts
          - *Sugars*

TABLE XV-2

Treatment of an Ether Extract from the Ether Extraction of a Freeze-Dried Plant Tissue (19)

- Extract (Lipids, pigments, organic acids)
  - Wash with sodium carbonate
    - Aqueous layer contains *organic acids*
    - Ether layer contains Pigments and Lipids
      Evaporate to small volume, distribute between petroleum ether and 90% ethanol
      - Alcoholic layer
        Lipids and *xanthophylls*
        The more polar components of the mixture
      - Petroleum ether layer
        Pigments of a less polar type

must, however, modify a scheme like this to suit the particular exigencies of his work. For example, some pigments are not ether-soluble, and thus will appear with the residue, in Table XV-1. Pursuing the scheme of this table, it should be recalled that ion exchangers are not merely such, but also adsorbents and sorbents, so that separations of naturally occurring mixtures (or any others, for that matter) with exchangers should be carried out with sharp attention paid to possible non-ion exchange behavior (Chapter IX, Sections V, VI, VII).

Examples of other approaches to the examination of tissues are very numerous. For example, Bradfield and Flood (103), examining the constituents of fruit plants, disintegrate the shoots and percolate the material with methanol at a low temperature. The methanolic extract, containing extracted water, is concentrated under reduced pressure to remove most of the methanol, and the resulting aqueous liquid is poured from a green sludge, the chlorophyll fraction. The remaining steps are tailored to the particular situation, e.g., to the removal of crude phlorhizin when apple shoots were studied, or arbutin, when pear shoots were studied, etc.

Partridge has proposed a scheme for handling aqueous extractives, and has referred to some of his own work (710–714) and that of others (126, 429,585,987) relevant to the problem.

The handling of animal tissues presents the same kinds of problems, in principle.

2. An Asphalt

O'Donnell has presented a scheme (692) applicable to the examination of an asphalt. This is used to illustrate some approaches that might be applicable to natural products. The scheme is shown in Table XV-3. One objective was to obtain fractions in which the spread of molecular sizes was narrowed down somewhat. These methods, as *B*-type procedures—molecular distillation, urea complexing, and thermal diffusion—may be extended to other kinds of separations.

3. Inorganic Mixtures

Inorganic mixtures are not discussed since the methods for getting them into solution are thoroughly treated in analytical texts.

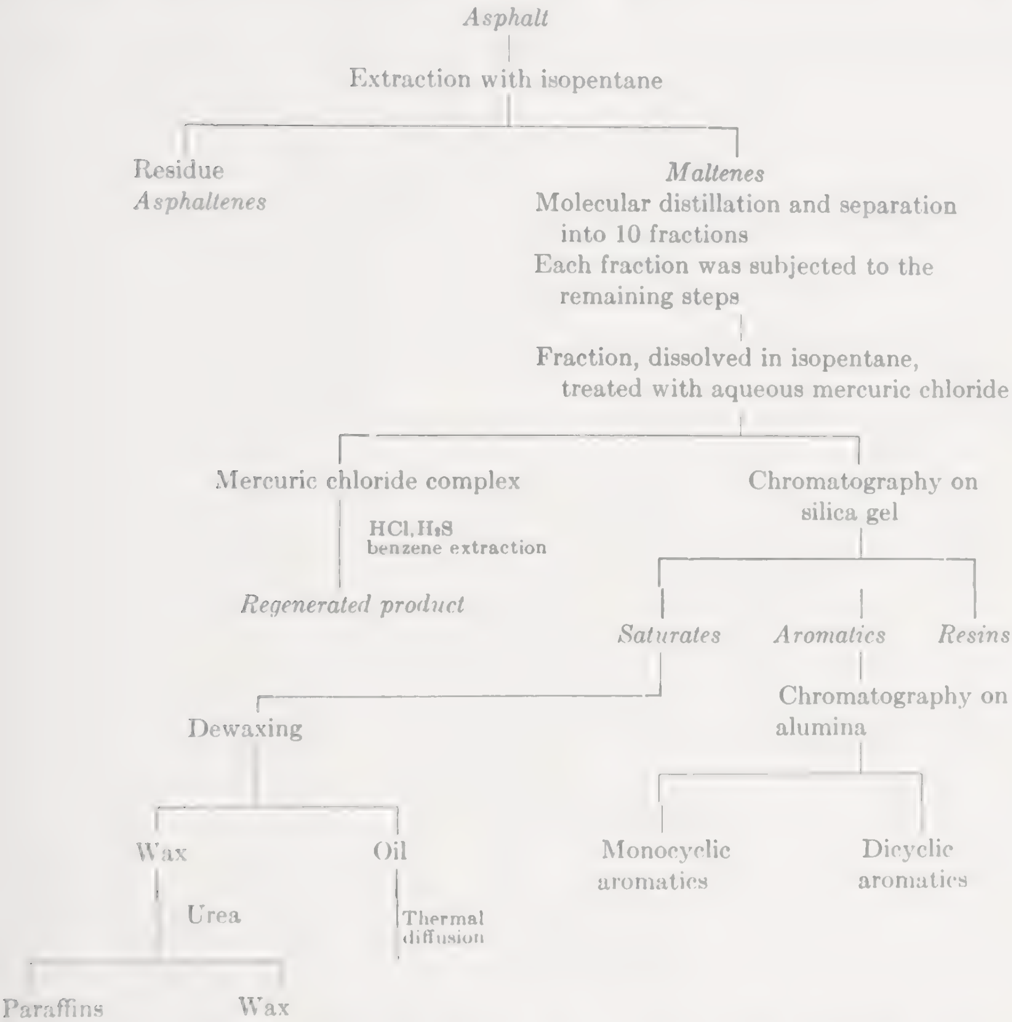
V. CHROMATOGRAPHY

1. Materials

It is useful to have on hand certain materials as a minimum stock with which to start the examination of a new problem. These may comprise

TABLE XV-3

Schematic Diagram of the Separation of an Asphalt into Fractions Suitable for Further Examination (692)



adsorbents: alumina, silica gel, silicic acid, Darco G-60 charcoal, U.S.P. calcium carbonate; ion exchangers: strong and weak acid and strong and weak base; papers in the form of sheets, smooth circular papers, and paper powder, a kieselguhr filter aid; organic and other reagents; and a variety of common polar and nonpolar organic solvents.

2. Little or No Experience in Chromatography

A person with little or no experience in chromatography should carry out several well worked out experiments to familiarize himself with the techniques. He might repeat some of the experiments given as examples

of procedure in the various chapters of this book, though these were not specially chosen to meet this purpose.

An easy and a very striking first experiment is the following (941). Several tapered strips of smooth filter paper are cut on the Rockland and Dunn pattern (Fig. VII-1) to fit 6-in. test tubes. At a point 0.6 cm from the narrow end and centered with respect to the edges of each strip is placed a *tiny* spot of green cake-coloring dye such as can be purchased in a grocery store. (These dyes are quite intense. They are sold usually in sets of four colors, any of which may be used in this experiment.) In the bottom of one test tube is put 0.5 ml. distilled water; in another 0.5 ml *n*-butanol. In a third tube put 0.5 ml. of a mixture of butanol–acetic acid (glacial)–water, 4:1:2 (v/v/v). The paper strips are introduced as shown in Fig. VII-1. The influence of the developers will soon become evident. The highly polar water moves the dye with the solvent front. The *relatively* nonpolar butanol hardly has an effect. The mixture produces several well-marked zones.

In another experiment, cellulose powder may be made into a slurry with the developer and poured into a chromatography tube (Fig. VI-2). After settling, the liquid is drained down just to the top of the column, which may be pressed down with the large end of a cork on the end of a rod or stiff wire. A few drops of cake-color, red, blue, yellow, for example, are dissolved in a little developer and applied to the top of the column, or are mixed with some cellulose powder and packed on top of the column. This is followed by developer. As development continues the formation, movement, and widening of zones are clearly visible.

3. Choice of System

If the mixture to be separated is volatile, gas partition chromatography is most highly to be recommended. It is simple, rapid, and versatile; the apparatus is simple to set up and operate. By volatile is meant having a boiling point below about 300°C. The method is particularly to be considered in new problems because the choice of phases is simplified: an inert gas serves as the mobile phase. Also the rules for predicting stationary phases seem relatively straightforward (Chapter V).

Very little can be said about foam and emulsion separation, except that where surface-active substances are involved they should be seriously considered—particularly foam separation, which is easy to handle and requires very little apparatus (Chapter XI).

The properties of electron exchangers are provocative, but little can be said about them that is germane here (Chapter X).

This leaves adsorption, partitions, and ion exchange methods. The last of these would naturally be one of the first explored for the separation of

ionic substances, though it may be useful with nonionic (Chapter IX). The adsorption and partition are most widely useful of all, being applicable to low and high molecular weight material, with all kinds of functional groups.

4. Trial Runs

Trial runs on a mixture may be made as follows. If there is any analogy in the literature it should be made use of, if only as a starting point. The choice of phases is made for *partitions* as indicated in Chapter XIV. Since results based on paper partition can usually be directly transferred to columns, or with minimum difficulty can be scaled up to columns, all preliminary work should be done with paper. The preliminary work can most easily be done using Rockland and Dunn's simple technique. A convenient procedure, if much exploratory work is continually being done, is to keep glass-stoppered test tubes ready prepared with common stable developer systems, and to cut paper as shown in Fig. VII-1,*d* from a roll of paper ribbon (592). The unstable solvent mixtures, 1-butanol–acetic–water, for example, cannot be kept, but are easily made up on the spot.

The most generally useful solvent system, it seems, is aqueous butanol (1-butanol with water). *If no hints are available that would lead to any other system, this should be tried first,* followed by the same system to which acetic acid, or ammonia, or benzene, etc., as needed is added. Generally, a mobile phase is chosen on the basis of information gathered during the working up of the mixture—the *B* steps of Sections III and IV, as explained in Chapter XIV, Section IV. In more difficult cases, as with high molecular weight and/or polyfunctional compounds, the very systematic working out of a binary or ternary phase diagram, as outlined by Porter and Martin, is the only practical procedure.

If *adsorption* chromatography seems like a likely method, the following procedure can be used. Small columns, prepared either in a medicine dropper or similar tube, or in a small-size tube such as shown in Fig. VI-21*b*, are prepared. *If there are no hints in the literature that would lead to another choice, alumina should be used first.* This is because it is an extremely versatile adsorbent, readily obtainable, and easily packed. It is best packed wet as a slurry in the developer or solvent to be used. This solvent should be the one used in working up the mixture, such as ether, or petroleum ether or benzene. Too polar a solvent should not be the first used.

A few drops of the mixture are allowed to run into the little column, which is inspected to determine if a zone is formed. If material breaks through the column readily, as determined by inspecting the effluent or making color tests on it, or by evaporating some of it, then the adsorbent

may not be strong enough, or the developer may be too polar, or in a favorable case some component of the original mixture was so poorly held that it could be simply separated from the rest. These possibilities are examined by qualitative tests, or by measuring the quantity put on the column and the quantity eluted, and comparing them. Sometimes merely evaporating a few drops of mixture on one watch-glass, and evaporating the effluent on another, comparing the residues, is enough to establish the situation.

Acidic materials are often separable on silica while not well separable on alumina. If the substances are colorless, then there is no reason for not trying them on charcoal, except that usually it must be mixed with a filter aid (paper powder or kieselguhr). Charcoal is particularly useful for fatty or waxy substances, which can be developed with benzene or the less toxic toluene, or with absolute ethanol.

When a suitable adsorbent has been found—one that seems to hold the mixture fairly well—and preferably one that produces some frontal analysis in the simple test experiment, the procedure can be scaled up to a larger operation.

This is done as follows. The small tube is packed with a known weight of adsorbent (the tube can be weighed before and after packing, if this is done dry). The mixture is then applied up to the break-through point. This tells how much of the mixture will be just held by the weight (or volume) of adsorbent used. Now a large column is packed with the weight (or volume) of adsorbent that would be required to hold back the amount of mixture that one wishes to work with, plus about 5 to 20 times as much adsorbent. When such a column is prepared, and the determined amount of mixture is applied to it, it can be confidently taken that a mixed zone of the calculated depth will have been formed at the top of the bed. This leaves 5 to 20 times as much adsorbent below the zone for the development to take place in. The zone is then developed either with the same solvent, if the adsorptives were not too tightly held as shown by the preliminary experiment, or with a gradient of increasing polarity.

As these experiments are carried out, with attention given to the special features of the adsorptive distribution that are described in Chapter VIII, Sections VI and VII, the pattern of behavior of the mixture will emerge and the procedures can be progressively refined.

VI. CONCLUDING REMARKS

This book has been constructed and written with two fundamental principles in mind. One is that all chromatographic phenomena have reasonable explanations which can be discovered. Often the discovery requires only the mobilization of knowledge from several areas of chemistry

or from some other science. At other times it may require finding through experiments, systematically planned, or based on a more subtle mobilization of knowledge.

The other principle was stated by P. Curie: "It is dissymmetry that produces phenomena." Chromatography is largely based on dissymmetry —on departure from ideality. A high degree of dissymmetry resides in a system of two immiscible phases. Perhaps this is what gives chromatographic methods their extraordinary power; perhaps it is why these tools are so acute and discriminating in their ability to separate mixtures. Perhaps, then, we should make the fullest use of this dissymmetry.

Appendix

I. SYMBOLS USED THROUGHOUT THE TEXT

Note that in some chapters a set of symbols will be defined specifically for local use, and the same letter may be used elsewhere with different meanings defined locally for use in that place. The symbols defined below are used throughout with the same meaning.

α Distribution coefficient. Ratio of equilibrium concentrations of solute (or adsorptive) in stationary phase to that in mobile phase, or function thereof:

$$\alpha = f\,(C_S^{\text{equil.}}/C_M^{\text{equil.}})$$

C_M Concentration in the mobile phase of the substance in question.

C_S Concentration in the stationary phase of the substance in question.

$C_M^{\text{equil.}}$ Equilibrium concentration of the substance in the mobile phase.

$C_S^{\text{equil.}}$ Equilibrium concentration of the substance in the stationary phase.

M Bulk mobile phase.

R An aliphatic or aromatic residue or group.

R Velocity of movement of the front edge of a zone in a column ÷ velocity of movement of developer in the tube above the column, measured in the same units, e.g., millimeters per minute.

R_F Distance from starting line to center of zone of substance ÷ distance from starting line to developer front, in paper chromatography.

For other symbols see Index.

II. SOURCES OF CHROMATOGRAPHY EQUIPMENT

This list is for the convenience of the reader. An endeavor has been made to make it comprehensive, but no guarantee of completeness can be given. No endorsement of any item or firm is implied in any way by this listing. Addresses of the listed firms are to be found in Section III of the Appendix.

Chromatography tubes, columns, racks for tubes
Ace Glass; Fisher & Porter; Harshaw Scientific; Kensington Scientific; LKB-Produkter; Microchemical Specialties; Phillips Scientific; Research Specialties; Schaar & Co.; Ivan Sorvall.

Column
Coupled column, Research Apparatus; coupled column, designed by Hagdahl & Danielson, Ivan Sorvall.

Densitometers
See Scanners.

Density balance, gas
Casella (Electronics); Martin, A. J. P.

Desalters
  Buch & Holm; Research Equipment; Shandon Scientific.
Dyes for standardizing alumina
  British Drug Houses.
Fraction collectors
  Autonomous Instruments; Gilson Medical Electronics; Harshaw; Kensington Scientific; Microchemical Specialties; Packard Instrument; Research Equipment; Schaar & Co.; Shandon Scientific; Ivan Sorvall; Technicon Chromatography; J. W. Towers.
Gas chromatography machines
  (See *Chem. Eng. News*, *34*, 1692 (April 9, 1956) for a survey of the early developments.); Beckman, Gas Chromatograph; Burrell, Fracton, Kromo-Tog; Cossor (Electronics); Consolidated Electrodynamics, Chromatograph; Fisher Scientific, Fisher-Gulf Partitioner; Griffin & George; Hallikainen Instruments, Hallikainen-Shell Chromatograph; Martin, A. J. P.; Perkin-Elmer, Vapor Fractometer; Podbielniak, Chromacon; Research Equipment, Distillograph; Wilkens Instrument & Research Inc., Aerograph.
Glass pipe
  Fischer & Porter.
Gryeksbo rolled filter paper column
  LKB-Produkter; Ivan Sorvall.
Heater plate, glass plate heater for paper strips or sheets
  Blue Ridge Glass, Ra-Grid Heater plate.
Interferometer applicable to chromatography
  American Instrument.
Paper chromatography equipment: jars, tanks, racks, trays, pipettes, cabinets, ovens, sprayers
  Kensington Scientific; Kopp; Microchemical Specialties; Research Equipment; Schaar & Co.; Shandon Scientific.
Micro and ultramicro burettes
  Emil Greiner (Gilmont ultra microburette); The Hamilton Company; Kopp.
Scanners, densitometers, for electromagnetic radiation, or radioactivity determinations
  Electronic Equipment (Export); Forro Scientific; Photovolt; Specialized Instruments, Spinco-Analytrol; Tracerlab; Unicam Instruments; W. M. Welch, Densichron.
Sprayers
  Matburn. See Paper chromatography equipment.
Thermal conductivity cells
  Gow-Mac Instrument Company.

III. ADDRESSES OF INDUSTRIAL FIRMS REFERRED TO IN TEXT

Note: Listing here implies no endorsement whatsoever.

Ace Glass, Inc., Vineland, New Jersey
Acheson Colloids Company, Port Huron, Michigan
Aluminum Company of America, 230 Park Avenue, New York 17, New York
Alupharm Chemicals, 322 Lafayette St., New Orleans, Louisiana
Amend Drug & Chemical Company, Inc., 117 East 24th Street, New York 10, New York
The American Colloid Company, Merchandize Mart Plaza, Chicago 54, Illinois
American Instrument Company, Inc., 8030 Georgia Avenue, Silver Spring, Maryland

American Nucil Company, Inc., 6301 Glidden Way, Jacksonville 8, Florida
Atlas Powder Company, Wilmington 99, Delaware
Autonomous Instruments, 241 E. Royal Forest Blvd., Columbus 14, Ohio
Baugh & Sons, 20 So. Delaware Avenue, Baltimore, Maryland
Beckman Instrument Company, Fullerton, California
Bell Telephone Laboratories, Murray Hill, New Jersey
Bio-Rad Laboratories, 800 Delaware Street, Berkeley, California
Blue Ridge Glass Corporation, Kingsport, Tennessee
British Drug Houses, Ltd., Poole, Dorset, England
Brown Company, Berlin, New Hampshire
Buch & Holm, Copenhagen, Denmark
Burrell Corporation, 2223 Fifth Avenue, Pittsburgh 19, Pennsylvania
Casella (Electronics) Ltd., 46/40 Osnaburgh Street, London, W. I.
Chemical Process Company, Post Office Box 829, Redwood City, California
Cliffs Dow Chemical Company, Marqette, Michigan
Columbia Chemical Division, Pittsburgh Plate Glass Co., Barberton, Ohio
Columbia-Southern Chemical Corp., 1 Gateway Center, Pittsburgh 22, Pennsylvania
Consolidated Electrodynamics Corporation, 300 N. Sierra Madre Villa, N. Pasadena, California
Davison Chemical Company, Baltimore 3, Maryland
The Dow Chemical Company, Midland, Michigan
E. I. du Pont de Nemours & Company, Wilmington, Delaware
The Eaton-Dikeman Company, Mount Holly Springs, Pennsylvania
Electronic Equipment (Export) Ltd., 101 Leadenhall Street, London E.C. 3
The Emil Greiner Company, 20-26 N. Moore Street, New York 13, New York
Filtrol Corporation, 3250 East Washington Blvd., Los Angeles 23, California
Fischer & Porter Company, Hatboro, Pennsylvania
Fisher Scientific Company, 717 Forbes Street, Pittsburgh 19, Pennsylvania
Floridin Company, Tallahassee, Florida
Food Machinery & Chemical Corp., Westvaco Chemical Div., South Charleston, West Virginia
Forro Scientific Co., 833 Lincoln St., Evanston, Illinois
Gilson Medical Electronics, 714 Market Place, Madison 3, Wisconsin
Gow-Mac Instrument Company, 100 Kings Road, Madison, New Jersey
Great Lakes Carbon Corporation, 18 East 48th St., New York 17, New York; Dicalite Division, P.O. Box 107, Walteria, California
Griffin and George, Ltd., Ealing Rd., Alperton, Wembley, Middlesex, England
Hallikainen Instruments, 1341 Seventh St., Berkeley 10, California
The Hamilton Company, 1134 Whitley Street, Whittier, California
H & V Specialties Inc., West Groton, Massachusetts
Harshaw Scientific, Cleveland 6, Ohio
Industrial Minerals & Chemical Company, Sixth & Gilman Sts., Berkeley 10, California
Johns-Manville, Manville, New Jersey
Kensington Scientific Corp., 98 Rimon Road, Berkeley 7, California
Kaye Laboratory Supplies, Inc., 1680 Second Ave., New York 28, New York
Linde Air Products Company, 30 East 42nd Street, New York 17, New York
LKB-Produkter Fabriksaktiebolag, Box 12035, Stockholm 12, Sweden
Martin, A. J. P., Abbottsbury, Barnet Lane, Elstree, Herts, England
Matburn, Ltd., 25 Red Lion Street, London, W.C. 1
Matthew Laboratories, 345 Nepperhan Avenue, Yonkers 2, New York

Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California
Minerals & Chemicals Corporation of America, Menlo Park, New Jersey
Monsanto Chemical Company, Inorganic Chemicals Division, St. Louis 4, Missouri
National Aluminate Corp., 6216 West 66th Place, Chicago 38, Illinois
National Carbon Company, 30 East 42nd Street, New York 17, New York
Packard Instrument Company, P. O. Box 428, La Grange, Illinois
Perkin-Elmer Corporation, Norwalk, Connecticut
The Permutit Company, 330 West 42nd Street, New York 36, New York
Phillips Scientific Corporation, 28 Carleton Street, Cambridge 42, Massachusetts
Photovolt Corporation, 95 Madison Avenue, New York 16, New York
Pittsburgh Coke & Chemical Co., Neville Island, Pittsburgh 25, Pennsylvania
Podbielniak, Inc., 341 East Ohio Street, Chicago 11, Illinois
H. Reeve Angel & Company, Inc., 52 Duane Street, New York 7, New York
Research Apparatus Company, 4643 Grand Avenue, S. Minneapolis, Minnesota
Research Equipment, 1135 Third Street, Oakland, California
Research Specialties Company, 1148 Walnut Street, Berkeley 7, California
Rohm & Haas Company, Washington Square, Philadelphia 5, Pennsylvania
E. H. Sargent & Company, 4647 W. Foster Avenue, Chicago 30, Illinois
Schaar & Co., 754 W. Lexington St., Chicago 7, Illinois
Schleicher & Schüll, Keene, New Hampshire
Shandon Scientific Company, Ltd., 6 Cromwell Place, London, S.W. 7
Ivan Sorvall, Inc., P. O. Box 230, Pearl Street, Norwalk, Connecticut
Specialized Instruments Corporation, Belmont, California
Tamms Industries, Inc., 228 No. LaSalle Street, Chicago 1, Illinois
Technicon Chromatography Corporation, Chauncey, New York
Tennessee Eastman Corporation, Kingsport, Tennessee
J. W. Towers & Company, Ltd., Victoria House, Widnes, England
Tracerlab, 130 High Street, Boston 10, Massachusetts
Unicam Instruments, Ltd., Arbury Works, Cambridge, England
U. S. Gypsum Company, Chicago, Illinois
W. M. Welch Manufacturing Company, 1515 Sedgwick Street, Chicago 10, Illinois
West Virginia Pulp & Paper Company, 230 Park Avenue, New York 17, New York
Wilkens Instrument & Research, Inc., Acheson Bldg., Berkeley 4, California

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